University of Alberta

Biological and chemical assessment of three weathered creosote contaminated soils: An ecotoxicological approach

by

Jeffrey William Adrien Charrois ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Master of Science**

in

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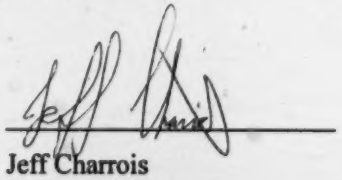

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Abstract

Creosote contaminated soils may pose a risk to environmental health because they contain a myriad of toxic compounds including polycyclic aromatic hydrocarbons (PAHs). Current government regulations focus on the concentration of contaminants and not bioavailability. An ecotoxicological approach evaluated three aged creosote, two slurry-phase biotreated, and two pristine control soils. The ecotoxicity testing included acute (14 day survival) and subchronic (DNA adduct quantification) bioassays using the earthworm *Eisenia fetida*. Soil characteristics and contaminant concentration data were considered in conjunction with the biological data, providing a holistic evaluation of toxicity compared to assessments based on biological or chemical data alone. Chemical and biological results suggest: 1) biotreatment does not always remove acute toxicity; 2) soils contaminated above current Alberta Tier I guidelines are not always acutely toxic; and 3) subchronic toxicity testing did not reveal the increases in DNA adduct formation in *Eisenia fetida* anticipated after exposure to elevated benzo[a]pyrene concentrations, or prolonged exposure to biotreated soils.

To Both My Families:
Theresa, Ray, Joan, Greg,
and the Walter Family

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List of Abbreviations

ATP – Adenosine Triphosphate

BaP – Benzo[a]pyrene

CCS – Core Contaminated Soil

DBT – Diobenzothiophene

DCM – Dichloromethane

DDW – Double Deionized Water

DNA – Deoxyribonucleic Acid

EC – Electrical Conductivity

GC / MSD – Gas Chromatography / Mass Selective Detector

LC_{50} – Lethal Concentration$_{50}$

NAPL – Non-aqueous Phase Liquid

NRC – National Research Council

PAH – Polycyclic Aromatic Hydrocarbon

RNA – Ribonucleic Acid

SIM – Selected Ion Monitoring

SOM – Soil Organic Matter

SPE – Solid Phase Extraction

TEO – Total Dichloromethane Extractable Organics

TLC – Thin Layer Chromatography

TN – Total Nitrogen

TOC – Total Organic Carbon

WFP – Water Filled Pore Space

Chapter 1: General Introduction

1.1 Introduction

With the growth of industrial activity comes the increase of environmental pollution. Concern about soil contamination has developed over the last decade into a major environmental issue (Sheppard et al., 1992). The boundaries between the environment and health sectors are beginning to overlap. Issues that are relevant to environmental health are also being recognized as personal health concerns. For example, answers to health questions regarding the toxicity of trace quantities of xenobiotics such as endocrine disrupting compounds, polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs), in the environment are becoming more critical. People are beginning to realize that pollution in their local environment ultimately has adverse consequences for their health as well as the health of other organisms that inhabit the global ecosystem.

When and how much to remediate as well as remediation strategies and endpoints have been debated among officials from government, industry, and academia. Ultimately, concerns relate to the level of risk humans are willing to accept for having a contaminant present in an ecosystem. The challenge is determining a method that adequately assigns risk to contaminants and subsequently allows for an informed risk management decision to be made. An ecotoxicological assessment allows for environmental risks to be assessed because several different components such as soils, contaminants and the biota are investigated.

1.2 Soil

Soils are dynamic living entities, which provide habitats and nutrients to many forms of life. The health of soils is an integral part of a sustainable ecosystem. As an essential component of the ecosystem, soils influence not only food and fiber production but also maintain environmental quality on scales ranging from local to global (Doran and Parkin, 1994).

Soils act as the great integrators of environmental processes. At the intersection of the atmosphere, biosphere, lithosphere, and hydrosphere is where soil develops. All four components are necessary for soil formation. Soils are under constant physical, chemical and biological change (Buol et al., 1980). The intimate role that soils occupy in the environment allows a contaminant, which is introduced into the soil, to be widely distributed to other environmental compartments via biogeochemical cycling of elements. Proper management of pristine soils as well as the adequate remediation of contaminated soils are necessary. Neglected soils will cease to be productive entities of the ecosystem.

The four main processes that develop soils are additions, removals, transformations, and translocations. These processes, in conjunction with the soil forming factors are responsible for the creation and development of soils on the landscape. The soil forming factors are: climate, organisms, topography, parent geological material, and time (Jenny, 1941). Humans can also be added to the list of soil forming factors. Soils are a three phased system: solid, liquid, and gas. It is these phases which set the framework for studying organism habitats and how soil components on a microscale can influence the availability of contaminants.

Since soils act as a gauge for environmental quality, an understanding of where soil comes from and how soils develop are both necessary components when evaluating soil health. The variable composition (chemical, physical, biological) and spatial distribution of soils must be considered when evaluating the distribution of contaminants within the soil profile (Eijsackers, 1994). Soil characteristics are known to influence the transport, toxicity, degradability and bioavailability of compounds (Grosser et al., 1995; CCME, 1996). Contaminants in the soil can be adsorbed onto surfaces such as mineral or organic particles, absorbed into mineral or organic particles, dissolved in soil water, or vaporized into soil gases (Eijsackers, 1994). Thus soil components need to be considered when characterizing a site because contaminant behavior can be greatly influenced by soil physicochemical and biological properties.

Soils at contaminated sites often do not reflect the concept of individual soils naturally occurring on the landscape, as described above. Rather, soils at industrial sites are often contaminated mixtures of various soil horizons as well as parent geologic material (PGM). Use of the term contaminated soil, in this thesis, refers to an admixture of contaminated soil horizons and PGM. Moreover, the term weathered, which often refers to "physical and chemical changes produced in rocks, at or near the earth's surface, by atmospheric agents" (SSSA, p.123, 1997) will be considered in a broader context throughout this thesis. Weathered as used here will encompass contaminated soil material that is aged at a site. Weathered also includes soil that is exposed not only to atmospheric elements at the earth's surface but also to additional environmental regimes such as ground water.

1.3 Hazardous Waste Sites

1.3.1 Overview

Soils can influence the health of humans and animals both indirectly and directly (Doran et al., 1996). Indirectly, soils can influence air, water, and food quality, which ultimately relate to public health concerns. Exposure to contaminated soil by ingestion, dermal contact, or inhalation of particles, can result in direct health concerns. In Canada there are over 10 000 contaminated sites, 1000 of them are "high risk" (Hrudey and Pollard, 1993). In all societies there are scarce resources and an infinite number of combinations in which to allocate such resources. The scarce resources in question are: money to pay for the clean up of a contaminated site as well as risk management resources. A second question that arises out of the first is: how clean is clean? Should every molecule of contamination be removed? This would likely be impossible. The lower the level of permissible residual contamination, the higher the total costs associated with the remedial action. Thus, a balance is required between economic costs to society and the risk of an overall contamination level in the ecosystem.

Ecological risk assessments attempt to answer the question of how clean is clean, and thus start to limit the potential expenditures associated with site

remediation. Ecological risk assessments are necessary because they attempt to identify risks associated with a given level of contamination. If there is no health risk, there may be no need to take any further remediation action.

There are various types of contaminated sites in Canada, ranging from brine spills and heavy metal contamination to oil well blow-outs and creosote contaminated sites. The focus of this work will be on creosote contaminated sites and the associated risk to environmental health.

1.3.2 Creosote Contaminated Sites/Soils

Creosote can refer to several different distillation products, which are used for a variety of purposes including insecticides, fungicides, preservatives, and pharmaceuticals (Von Burg and Stout, 1992). Sources for distillation include wood (beechwood creosote), sap from the creosote bush, and coal (coal tar creosote). For this work, the term creosote will refer to coal tar creosote.

Coal tar creosote is a complex mixture of several different chemical classes dissolved in a petroleum carrier (Pollard et al., 1993). Each class, when analyzed by gas chromatography-mass spectrometry (GC/MS), will yield hundreds of identifiable compounds (Guillén et al., 1992) along with many substituted compounds for which authentic standards do not currently exist. As a result, creosote is a mixture that can differ in chemical composition from one site to another. The major chemical classes found in creosote are: polycyclic aromatic hydrocarbons (PAHs) (85%); phenolic compounds (10%); and N, S, and O-heterocyclics (5%) (Mueller et al., 1989). Potential adverse environmental health risks from exposure to creosote are a concern because of the known acute and chronic toxicity associated with many of the individual components found within the mixture, such as the PAHs.

1.3.3 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous within our ecosystem and can persist as toxicants in the environment (Shuttleworth and Cerniglia, 1995). PAHs are the result of incomplete combustion of organic materials including coal tar and petroleum products (Grosser et al., 1995; Larsen, 1995). In the

environment, PAHs occur within complex mixtures such as creosote with hundreds of different compounds (Mueller et al., 1989). The major creosote constituents of interest, for this thesis, are illustrated in Figure 1.1. These constituents were chosen because authentic primary standards were available. Further, the 16 PAHs are on the United States Environmental Protection Agency's list of priority pollutants. The 16 priority PAHs are a small subsample of the entire PAH class, however, they do represent a range a different chemical characteristics.

Concern with exposure to PAHs arises because PAHs, such as benzo[a]pyrene, are recognized animal carcinogens (Larsen, 1995) and genotoxic agents (Shuttleworth and Cerniglia, 1995). Benzo[a]pyrene can be metabolized into reactive species that covalently bind to macromolecules such as DNA. Bacteria oxidize PAHs via a dioxygenase to form cis-dihydrodiols, whereas fungi (Atlas and Cerniglia, 1995), earthworm (van Schooten et al., 1995) and mammalian systems (Parkinson, 1996) potentially can use monooxygenases such as the cytochrome P-450 system, yielding a trans-dihydrodiol. It is the trans isomer, benzo[a]pyrene 7,8-dihydrodiol-9,10-epoxide, which is the ultimate carcinogenic metabolite of benzo[a]pyrene in mammalian systems (Atlas and Cerniglia, 1995). In earthworms, it is unclear how BaP is activated. BaP activation could be by cytochrome P-450, however, soil and/or intestinal microbes may also be involved.

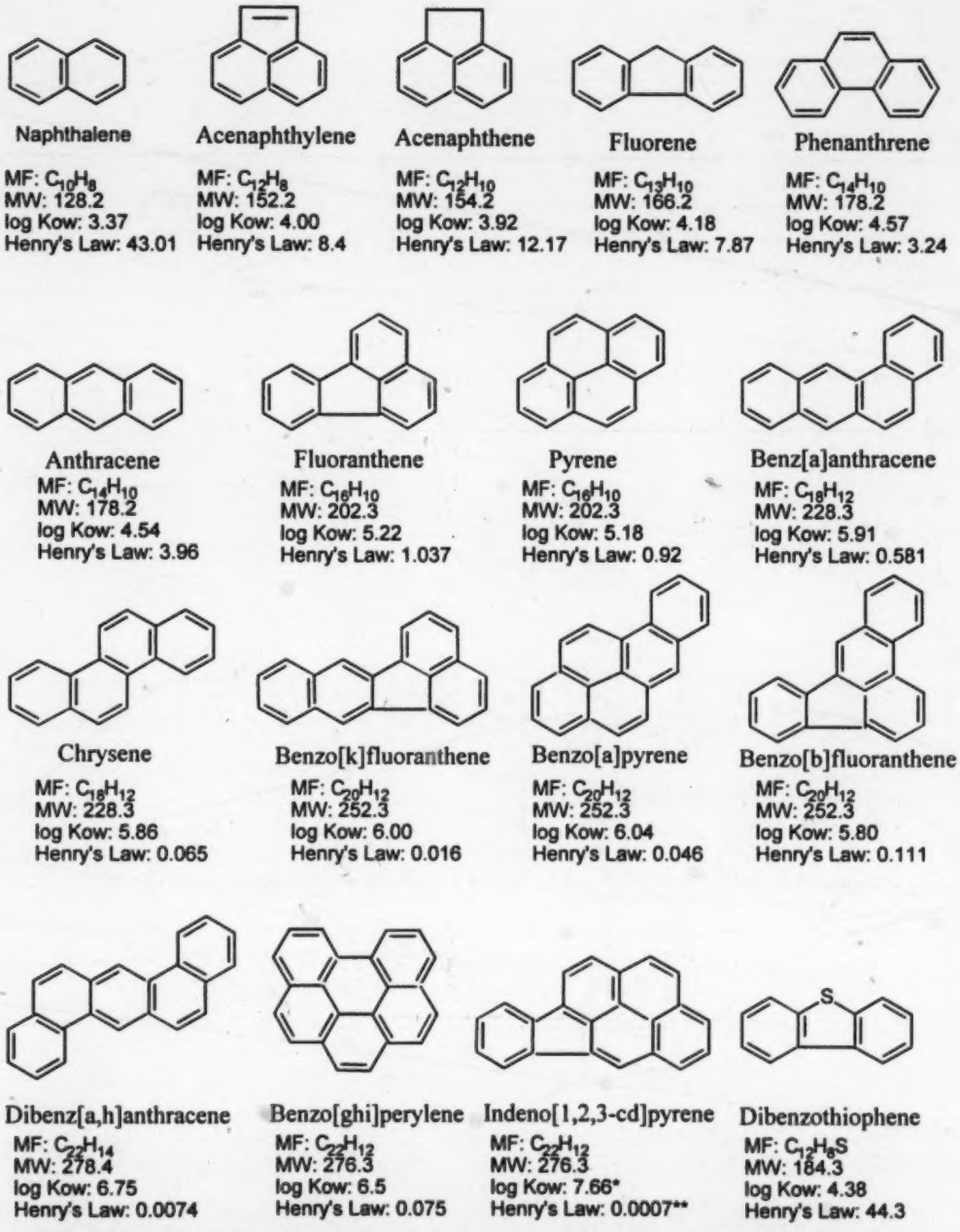

Figure 1.1: Common constituents of creosote. Summary of physical-chemical properties at 25°C. All values are from Mackay et al. (1992a and 1992b) except*, ** (Smith et al., 1995); (converted from atm m^3 $mole^{-1}$). Units for Henry's Law are Pa m^3 $mole^{-1}$.**

1.3.4 Ecotoxicology

In order to prioritize the multitude of existing contaminated sites as well as future sites, a suitable framework that characterizes and assesses risk must be utilized. An ecotoxicological approach can be used to evaluate environmental health concerns. Ecotoxicology is a branch of classical toxicology that focuses on the fate and adverse interactions between toxic substances and the ecosystem (Kendall et al., 1996). Using the principles of ecotoxicology, chemical components and biological responses to contaminant mixtures are assessed. An ecotoxicological approach to evaluate environmental risk should also consider the medium that is contaminated, because the environment is heterogeneous and the fate and behavior of contaminants will be influenced by site characteristics such as soil. Many other site variables can be important in regulating toxicity, however this thesis focuses on the soil environment.

Currently, Alberta Tier I guidelines focus entirely on the concentration of contaminants in the soil. Regulation of organic contaminants based on solvent-extractable concentrations may be assigning inappropriate levels of risk to contaminants in the soil (Hund and Traunspurger, 1994; Loehr and Webster, 1996). Several criticisms exist regarding the strict reliance on solvent-extractable data to predict environmental risks. Lambolez et al. (1994) cite the difficulties in identifying certain micropollutants within a complex waste mixture and predicting synergistic or antagonistic interactions between pollutants as reasons why toxicological studies need to complement chemical analysis. Site history can dictate which classes of compounds will be screened for in a site assessment. Failure to quantify all compounds along with unconsidered degradation metabolites will limit the usefulness of regulations based on solvent-extractable contaminants (Hund and Traunspurger, 1994). Further, the toxicity of aged soil contaminants seems to be less than would be predicted by chemical analysis (Alexander, 1995). Strict reliance on chemical data without complementary biological testing compromises regulatory guidelines, remediation objectives, and ultimately environmental health by over- or underestimating the risk which contaminants pose to organisms.

1.4 Scope

There are five chapters in this thesis. Specific objectives are detailed for each of the three experimental chapters. In Chapter 2, soil master variables (Alder et al., 1994) as well as contaminant concentrations, before and after slurry phase biotreatment are documented. Total dichloromethane extractable organics and PAHs were characterized in detail. Chapter 3 deals with acute toxicity as a result of earthworm (*Eisenia fetida*) exposure to creosote contaminated soils. Toxicity testing was conducted on whole soils. The concepts of 60 % water filled pore space, earthworm-days and PAH / TEO ratios are introduced to supplement current practices in acute ecotoxicological testing. Chapter 4 focuses on subchronic toxicity testing. DNA adducts provide a measure of exposure to PAHs and thus a measure of bioavailability. A synthesis of the entire investigation, including implications of the results as well as suggestions for future research, is reported in Chapter 5.

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Chapter 2: Chemical Analysis and Soil Characterization

2.1 Introduction

Proper site characterization is essential for identifying hazards at a contaminated site. Moreover, chemical analyses are required for identifying the types and amounts of contaminants in a soil. Determining the magnitude of exposure to contaminants is also critical in the chain of events starting from the release of an environmental pollutant into the environment and eventually leading to a disease state (Larsen, 1995). The relative contribution of each source of a pollutant must be accounted for if realistic exposure scenarios are going to be developed (Larsen, 1995). The predictive powers of models and risk assessments rely on quality chemical data in order to be valid.

Historically, and even today, chemical concentration endpoints have been the cornerstone of regulatory guidelines and, therefore, have driven the initiation and termination of the remediation process. For example, environmental regulations often rely solely on chemical extraction procedures for organic contaminants when determining permissible concentrations of hydrocarbons in the soil (Alberta Environmental Protection, 1994). Regulations require quantification of the oil and grease fraction to estimate the extent of hydrocarbon contamination (Pollard et al., 1992). The Soxhlet extraction method estimates the oil and grease component of a contaminated soil by using a solvent, such as dichloromethane, to indiscriminately remove soluble compounds. The solvent is then evaporated off and the extractable fraction is gravimetrically determined. Thus, the oil and grease or total extractable organic (TEO) limit is only a gross measure of contamination and provides limited information for assessing the feasibility of a biological treatment system or the actual toxicity of a hydrocarbon contaminated soil to an organism (Hrudey and Pollard, 1993).

Further characterization of specific classes of compounds such as mono-, poly-, and hetero-cyclic aromatics within the oil and grease fraction is necessary when attempting to assess the risk contaminated soils pose to the health of the environment.

In order to quantify many of the hydrophobic constituents in an oily sample, a method of analysis that can separate constituents from an oily mixture is required. Several analytical options are available to quantify the constituents dissolved in oily extracts from soils contaminated with creosote. The use of gas chromatography-mass spectrometry (GC/MS) is one of the most powerful techniques for the study of PAH mixtures in coal tar pitch (Guillén et al., 1992). In this thesis, gas chromatography coupled with a mass selective detector was used to quantify creosote constituents.

Polycyclic aromatic hydrocarbons (PAHs) are a group of hazardous organic chemicals that occur ubiquitously in environmental samples. They are usually present within complex mixtures such as creosote (Cerniglia, 1992). PAHs constitute 85% (w/w) of creosote (Mueller et al., 1989). Some of the PAHs, such as benzo(a)anthracene and benzo(a)pyrene and their metabolic by-products are known genotoxic and carcinogenic agents (Cerniglia, 1992). The risk PAHs present to organisms in the environment is one reason why PAH concentrations are regulated. Often times it is the PAH concentration that must be decreased before a contaminated site is considered remediated. Since PAHs: make up such a large percentage of the total weight of creosote; some are known carcinogens, mutagens and genotoxic agents; and the concentration of them is regulated in the soil; their quantification is necessary.

It is also critical to describe the site characteristics, such as soil properties, which can mediate hazardous substance fate and behavior on a macro-scale or the bioavailability of a compound on a micro-scale. The Canadian Council of Ministers of the Environment (CCME, 1996) have identified some key soil variables that are considered to play important roles in contaminant behavior. The master variables of the soil are: clay content, redox potential, pH, and organic matter content (Alder et al., 1994). Soil parameters must be considered because they can influence the availability and degradation of compounds (Grosser et al., 1995). The quantity of non-aqueous phase liquids (NAPL) is also important to consider because of the NAPL's ability to control contaminant partitioning (Smith et al., 1995).

2.2 Objective

The objectives of the work reported in this chapter were twofold. Firstly, to characterize, chemically and physically, the test soils used in acute and subchronic toxicity testing, in terms of contaminant composition and soil master variables. Secondly, to document changes in contaminant concentration and distribution following 52 days of slurry phase biotreatment of two weathered creosote contaminated soils.

2.3 Materials and Methods

2.3.1.1 Soils

During October 1994 the three core contaminated soils (CCS) used throughout the work presented in this thesis were collected by Drs. P.M. Rutherford (Department of Renewable Resources) and D.K. Banerjee (Department of Chemical and Materials Engineering), both formerly at the University of Alberta. Processing of the contaminated soils took place throughout the fall of 1994. Several buckets, approximately 100 – 150 kg of each field moist soil, were collected and subsequently processed (Section 2.3.1.2) at the University of Alberta's Research Station (Ellerslie, Alberta). Use of the term contaminated soil, in this thesis, refers to an admixture of contaminated soil horizons and parent geological material (PGM).

All soils described in this section will be referred to throughout the entire thesis. The three CCS were collected from two weathered creosote sites and were subsequently characterized (this Chapter). The first CCS collected was from an industrial site in Edmonton, Alberta (EDM) which contained admixed surface and subsurface material. The second CCS was sampled from the subsurface at a site in Prince Albert, Saskatchewan (PAC). The final CCS (PAL) was from the same site at Prince Albert, however, it was sampled from a different location on site and had lower concentrations of total dichloromethane extractable organics (TEO) and total PAHs compared to PAC. The contaminated core soils were chosen to reflect a range of soil and contaminant characteristics (Table 2.3). Because no appropriate control soils were at or near the contaminated sites, two pristine control soils were selected to

approximate as many master soil variables (Alder et al., 1994) as possible of the contaminated core soils. The master variables evaluated were texture, organic carbon content, pH, and electrical conductivity (EC).

The two pristine soils were collected in May 1997. The pristine control soil (Malmo-C) used for EDM was sampled from a C horizon from the Malmo soil series located at the University of Alberta Research Station (Ellerslie, Alberta; NE - 24 - 51 - 25 - W4). The Malmo soil is an Eluviated Black Chernozem, which developed on lacustrine parent material (Bowser et al., 1962). The pristine control soil (Brud-C) used for the PAC and PAL soils was sampled from a C horizon from the Dune Sand series located north of Bruderheim, Alberta (NE - 20 - 56- 17- W4). The Dune Sand series is an Orthic Regosol, which developed on aeolian parent material (Bowser et al., 1962). The site is located on Range Road 204, approximately 2 km south of Highway 38, between TWP Rd 562 and Highway 38.

2.3.1.2 Processing

The CCS were originally too wet to sieve, so they were spread out on plastic sheets in a thin layer and allowed to dry slowly in a machine shed. As the soils were drying, they were mixed frequently, ensuring the soils did not totally dry out. After sufficient drying (3 – 13 g H_2O per 100 g oven dried soil, depending on texture) (Rutherford et al., 1998), the soils were passed through a 4 mm sieve. Each soil was thoroughly mixed by hand tools and then homogenized in a clean cement mixer for 30 min. Once the mixing was complete, the mixer was washed and the next soil was homogenized. The portion of soil that did not pass through the 4 mm screen, was termed coarse fragments and stored separately from the homogenized soil (< 4 mm). Approximately 8 % (by weight) of the EDM soil was coarse fragments. The physical handling of all the contaminated soils may have resulted in the loss of some volatile components however all soils were handled in a similar manner. All soils were then stored in sealed 20 L plastic buckets at 4°C until use. In the spring of 1997, when toxicity testing began, the homogenized contaminated soils were passed through a 2 mm sieve (No. 10 particle size fraction; Canadian Standard Sieve Series/W.S. Tyler Co., St. Catharines, ON). Coarse fragments (> 2 mm) were discarded.

The two pristine soils were processed in a similar manner as the CCS, except the soils were slowly dried in a fumehood at 22°C, and they were mixed by hand tools every few hours to facilitate uniform drying. All clods were physically broken and the soil was originally passed through a 2 mm sieve. The < 2 mm fraction was further homogenized by rolling each soil in a bucket for 10 – 15 min. The soils were kept in 20 L plastic buckets and stored at 4°C until needed.

2.3.2 Soil Characterization

2.3.2.1 Soil Chemical Analyses

Soil chemical analyses were conducted in duplicate on air dried samples, which had been passed through a 2 mm sieve. Values are reported as the average of replicate samples. Soil pH and EC were measured using the same fixed ratio suspension of double deionized water (DDW) and air dried soil in a 2:1 solution:soil ratio (mass basis). Soil pH was determined using a pH meter (Fisher Accumet® Model 630, Fisher Scientific, Pittsburgh, PA) with a glass electrode (Hendershot et al., 1993). Electrical conductivity was measured (Rhoades, 1982) with a YSI Model 31 Conductivity Bridge (Yellow Springs Instrument Company Inc., Yellow Springs OH).

Total organic carbon (TOC) and total nitrogen (TN) concentrations were determined using a Carlo Erba Strumentazione NA 1500 Nitrogen/Carbon/Sulfur Analyzer (Carlo Erba Strumentazione, Milan, Italy). Sample preparation included grinding air dried soils for three minutes using a Mixer Mill (Model MM2; Brinkmann Instruments Co., Rexdale, ON), until the sample reached a talc-like consistency. Inorganic carbon was removed before analysis from samples high in carbonates with the addition of 6 M HCl. Soil samples were auto-injected into an oxidative furnace tube (1020°C) that contained chromium oxide, to enhance oxidation, and silvered cobaltous cobaltic oxide, to remove halides. Within the combustion reactor the carrier gas was helium. As the sample was dropped into the chamber, a pulse of oxygen initiates a dynamic flash combustion of the sample (1800°C). The sample passes through a reduction tube of reduced copper and all N

and C atoms are converted to N_2 and CO_2 respectively. Next, the sample stream passes through a magnesium perchlorate column, to remove any water. The sample passes through a gas chromatograph column and is quantified using a thermal conductivity detector. Total organic C and total N concentrations were determined for contaminated and pristine soils, before and after 16 hours of Soxhlet extraction using 200 ml of dichloromethane (Section 2.3.5). Values of TOC and TN were reported as percentages of oven dry soil mass (105°C for 24 hours).

2.3.2.2 Soil Physical Analyses

Soil physical analyses were conducted on air dried samples, which had been passed through a 2 mm sieve. Soil particle size distributions were determined in duplicate for samples of all core contaminated and pristine soils using the hydrometer method (Sheldrick and Wang, 1993). The Malmo-C soil required a pretreatment of 0.01 M HCl to remove carbonates; all other soils remained untreated.

All soil water contents were gravimetrically determined, in triplicate, with a drying oven at 105°C as detailed by Topp (1993). Water contents were recorded as a percentage of oven dry soil mass.

2.3.3 Slurry Phase Biotreatment

The slurry phase biotreatment experiment was designed by Dr. P.M. Rutherford (formerly of the Department of Renewable Renewable Resources) and maintained in conjunction with A. Hashimoto (Department of Biological Sciences) at the University of Alberta.

Two of the contaminated soils (EDM and PAC) were selected for their potential to be biotreated based on previous work completed at the University of Alberta (Rutherford et al., 1998). Thirteen reactors for the EDM soil and eight reactors for the PAC soil were set up for 7.5 weeks (52 days) of slurry phase biotreatment as described by Rutherford et al. (1998). Reactors consisted of 2 L mason jars, which had perforated lids covered with filter paper that permitted air exchange.

Each reactor was inoculated with a subsample of soil slurry that was previously biotreated for 1 month (13 g of slurry for EDM and 10 g of slurry for PAC). The inoculation helped to initiate biodegradation as well as provide a soil microbial population that was already adapted to the specific set of environmental conditions (temperature, light) used in the biotreatment of weathered creosote contaminated soils.

A nutrient stock solution was added (Table 2.1) to the contaminated soil, along with additional water to produce a slurry (Table 2.2). Previous work (Rutherford et al., 1998) indicated that optimal soil mixing within the reactors occurred at soil:solution ratios of 1:1 for EDM and 3:1 for PAC. The actual soil:total solution ratios achieved were 0.88:1 for EDM and 2.3:1 for PAC, based on clean oven dried soil. Experimental protocols were designed to enhance the mixing conditions, maintain an aerobic environment, and maximize the soil mass treated per reactor. Overloading the reactor with soil, which could potentially result in the leakage of slurry out of the reactor, was avoided. Additionally, relatively low soil:liquid ratios were desired to increase soil batch volumes, particularly if a larger scale process was to be made feasible

Table 2.1: Nutrient stock solution used in the slurry phase biotreatment of EDM and PAC soils to obtain a N:P:S ratio of 10:0.7:0.1 (mass basis). The pH of the solution was adjusted to 7.2 with 10 N KOH.

Nutrients	Concentration ($g\ L^{-1}$)
NH_4NO_3	28.60
KH_2PO_4	1.46
K_2HPO_4	1.87
K_2SO_4	0.54

Table 2.2: Soil and nutrient data used in slurry phase biotreatment.

Soil	Mass of soil (g) (bucket moisture content†)	Volume Nutrient Stock Solution (ml)	Volume H_2O (ml)	Total Liquid Volume‡ (ml)
EDM	140	12	121	148.9
PAC	140	10	44	58.2

†Soil bucket moisture contents at the time of the experiment were 12% in EDM and 3% in PAC.
‡Includes the original soil water at bucket moisture content.

Based on the contaminant concentrations and nutrient additions, a designed C/N ratio of 15 was used in this experiment. Biotreatment reactors were placed horizontally on the roller bottle apparatus, kept in the dark at 22°C and continuously rotated (3.7 rpm) for 52 days. Vessels were observed throughout the experiment to ensure the experimental conditions were being maintained. Water loss was monitored throughout the treatment phase by weighing the reactors. Water was added to the reactors once, approximately halfway through the experiment, to maintain constant moisture contents.

2.3.4 Soil Standards

Two sets of standards were included in the chemical determination protocol (Section 2.3.5), each differing in source of contamination and texture. A primary marine sediment standard was obtained from the National Research Council of Canada (NRC), (Institute for Marine Biosciences Halifax, NS). The marine sediment was collected from a harbour in Nova Scotia and certified to contain specific quantities of 16 PAHs (Table 2.5). The sediment was freeze-dried and passed through a No. 120 (125 μm) sieve. This standard will be referred to as the primary standard. A secondary standard was also used. The secondary standard was a subsample of the PAC soil, which was air dried in a fumehood, sieved to less than 2 mm, homogenized on the roller bottle apparatus (3.7 rpm) for 7 days, and freeze dried to remove any remaining moisture. The secondary standard was included because it more closely represented the type of contaminated matrix, than did a marine sediment standard. This secondary standard, however, did not have a known contaminant distribution and served for quality control purposes only.

2.3.5 Soxhlet Extraction / TEO Determination

All solvents used were Optima grade (Fisher Scientific, Fair Lawn, NJ) unless otherwise stated. All glassware used for the Soxhlet extraction procedure was solvent-washed before use with three separate solvents: hexane, acetone, and dichloromethane (DCM).

Initially all flasks for the Soxhlet extractions as well as volumetric flasks were washed with 8 M ethanolic KOH and allowed to dry in an oven at 260°C. The glassware was then treated with a 5% solution of dimethyldicholorosilane in toluene (Sylon-CT, Supelco, Bellefonte, PA) as described in the method provided by the manufacturer. Sylon-CT was used to make the glassware inert, by deactivating binding sites on the glassware.

After each series of extractions, the Soxhlet glassware was solvent-rinsed with redistilled DCM, washed with soap and water, rinsed with DDW, and then stored in an oven at 260°C overnight to drive off any water or solvent remaining from the glassware. Before the next series of samples were processed the glassware was triple-solvent-rinsed, as described above.

The total dichloromethane extractable organic (TEO) content was determined gravimetrically (McGill and Rowell, 1980) as modified below. Approximately 25 g of air dried soil was placed in a single thickness cellulose extraction thimble (30 mm x 80 mm; Whatman®), mixed with approximately 10 g of $NaSO_4$, and extracted with DCM. To the soil, 2 ml of 53.5ng ml^{-1} of deuterated benzo[a]pyrene (D_{12}, 98 %; $C_{20}D_{12}$; Cambridge Isotope Laboratories, Inc) (BaP-d_{12}) dissolved in isooctane was added to the surface. The solvent was allowed to evaporate, and the soil was mixed in the thimble using a metal soil spatula. The BaP-d_{12} was an internal standard for the subsequent GC / MSD determination of PAHs from the Soxhlet extracts (Section 2.3.6). Dichloromethane (200 ml), in round-bottom flasks with two previously DCM extracted boiling chips (Hengar Granules, Hengar Co.), were heated using individual electric mantles (PL 312 Minitrol Glas-Col) to 10 - 12 cycles per hour for 16 hours in a Soxhlet extraction apparatus. The boiling chips were not Teflon ™ coated and thus there was the potential to sorb analytes of interest.

After the Soxhlet extraction was complete, DCM was distilled off at 60°C using a Rotovap, until 5 to 10 ml of extract remained. The extract was transferred to a 50 ml solvent-rinsed volumetric flask and made to volume with new DCM. The sample was split into two portions using a solvent-rinsed glass volumetric pipette. The first portion of the extract (25 ml) was placed in a pre-weighed aluminum

weighing dish and placed in a fumehood at room temperature. The pans were gently agitated to break any oily films that initially formed on the surface. The pans with the oily extract were subsequently re-weighed after 24 hours. The mass of the extracted material was determined gravimetrically (TEO or Total Oil and Grease) after removing the dichloromethane and expressed as a percentage (TEO g 100 g^{-1} of oven dry soil).

The second portion of extract was archived at 4°C for clean up and PAH quantitation by GC/MSD (Section 2.3.6.1). The extracts were stored in solvent rinsed 15 ml amber screw top vials with 18 mm solid cap PTFE liners (Supelco).

2.3.6 PAH Quantification

All soils used in the investigation were extracted with dichloromethane to gravimetrically determine the total extractable organic content (TEO) (Section 2.3.5). Quantities of 16 target PAHs and dibenzothiophene, a sulfur substituted heterocyclic compound, were quantified from an aliquot of the TEO extract by GC/MSD. The following creosote constituents were quantified for all soils: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, chrysene, benzo[k]fluoranthene, benzo[a]pyrene, benzo[b]fluoranthene, dibenz[a,h]anthracene, benzo[ghi]perylene, indeno[1,2,3-cd]pyrene, and dibenzothiophene.

2.3.6.1 Extract Clean Up Procedure

Creosote contains hundreds of individual chemical components from a diverse group of chemical classes such as: PAHs; phenolics; N-, S-, and O- heterocyclics; (Mueller et al., 1989) as well as saturates and asphaltenes. Compounds from different classes and even amongst the same class can interfere with each other when quantifying compounds of interest using GC / MSD. A Solid Phase Extraction (SPE) procedure was used to separate the desired nonpolar analytes from the interfering compounds of greater polarity in the Soxhlet extract. The SPE procedure allows PAHs to adsorb on the cartridge. Interfering compounds with higher polarity are not retained by the packing. Additionally, choice of elution solvents with different

polarities allows selective elution of analytes of interest. The extract clean up procedure was used so that the analytes of interest could be separated more clearly, thereby providing enhanced compound sensitivity for the GC / MSD system. The Soxhlet extract clean up procedure is detailed by Zemanek (1994), and is summarized below.

A 1 g / 6 ml LC-Florisil (magnesium silicate 100/120 mesh) cartridge (Supelco) was conditioned with 2 column volumes of DCM and allowed to air dry for 10 minutes. Then a 1 ml aliquot of the concentrated Soxhlet extract was applied to the surface of the Florisil packing. The extract was then eluted with 3 column volumes of DCM ensuring that the surface of the Florisil did not dry out. The eluate was collected in glass vials and stored at 4°C until all samples were cleaned up and the samples could be processed further. Some samples were more highly contaminated than others (PAC 100 %, PAC 50 %, EDM 100 %, EDM 50 %, and Std-2), which could potentially leave analytes of interest on the column. For such samples, a second elution of three additional column volumes of DCM was passed through the cartridge to remove any remaining analytes that were present after the first elution. The eluate was combined with that of the first elution and the samples were concentrated using N_2 blow down, as described later. As well, there were some third elutions, using one column volume of DCM, and fourth elutions, using one column volume of toluene, for the samples that were highly contaminated. Samples that were considered to have lower contamination had a second elution carried out as above but the elutions were not combined into one sample. The second elutions for the less contaminated samples along with the third and fourth elutions of the highly contaminated samples were analyzed by GC / MSD to determine if any PAHs remained on the SPE cartridge after the initial elution(s) of the Florisil cartridge.

After all the samples had been processed through a Florisil cartridge, the eluate was concentrated to a volume of 0.5 ml by N_2 blow down in a solvent rinsed glass volumetric centrifuge tube. Approximately 10 ml of extract at a time was added to a solvent rinsed volumetric centrifuge tube. The centrifuge tubes were placed in a heating block on top of a hot plate at a temperature of 40 – 45°C. Nitrogen gas was

passed over the surface of the eluate. As the volume was reduced, additional portions of each sample were added to the centrifuge tubes using a Pasteur pipette. The evaporation continued until a volume of 0.5 ml was reached. Adding 1 ml of toluene to the concentrated eluate provided a solvent for the PAHs to exchange into. The solution was concentrated by N_2 blow down until a final volume of 1 ml was reached. The solvent exchange was performed to allow better storage and handling of the final eluate. The final eluate was transferred into a solvent rinsed 2 ml screw top GC vial with PTFE lid (Supelco) and stored at 4 °C to reduce volatilization of the eluate.

2.3.6.2 Gas Chromatography / Mass Selective Detector

PAHs were quantified using a selected ion monitoring (SIM) method with a Hewlett Packard 5890 gas chromatograph and a Hewlett Packard 5970 mass selective detector (GC / MSD). The column was an HP-5; (5 %)-diphenyl-(95 %)-dimethylsiloxane copolymer, 30.0 m in length with an internal diameter of 0.25 mm and a film thickness of 0.25 µm. The carrier gas was helium, with column head pressure of 21 kPa. The solvent delay for each run was 5 minutes. Samples were injected using an autosampler, each sample injection was 2 µl. Zemanek (1994) optimized each sample run for peak abundance and separation starting with an oven temperature of 70°C, which was held for 4 minutes, followed by a thermal ramp of 10°C per minute to a temperature of 280°C, which was held for 13 minutes. The final temperature ramp was 4°C per minute to a temperature of 300°C, which was held for 5 minutes. The total run time for each sample was 48 minutes. A selected ion monitoring method was used to quantify each compound of interest. A major ion was selected for quantification along with a qualifier ion, which was used as confirmation for each compound of interest. Selected ion monitoring provided a method of reducing the interference from compounds that eluted at the same time as the compounds of interest.

2.3.6.3 Soxhlet Extraction Efficiency

Soxhlet extraction efficiency was determined by extracting a primary sediment standard (HS-3) which was obtained from the National Research Council (NRC) and comparing the certified PAH concentrations with the measured PAHs.

2.3.6.4 PAH Standards and Calibration

A mixture of 16 PAHs, dibenzothiophene, and BaP-d_{12} were used to construct a calibration curve to quantify compounds of interest that were contained in the Soxhlet extract. The original concentration of each PAH in the neat mixture was 2000 $\mu g\ ml^{-1}$ (Lot No. L-0831 Exp 7/99, Ultra Scientific). PAHs were dissolved in a 1 ml solution of DCM:Benzene (1:1). The dibenzothiophene (DBT) (≥ 98 %; Fluka Mississauga, ON) was dissolved in DCM to produce an original concentration 2024 $\mu g\ ml^{-1}$. The BaP-d_{12} (98 %) (Cambridge Isotope Laboratories, Andover, MA) was 2000 $\mu g\ ml^{-1}$ dissolved in isooctane. The calibration series consisted of 0.1, 0.5, 1.0, and 10.0 $\mu g\ ml^{-1}$. Using a calibration curve, which was updated before each run, enabled the operator to monitor the performance of the instrument and compensate for minor differences in the day to day detection of compounds. Each peak of interest in the chromatogram was selected manually and then integrated to determine the area under the peak.

2.3.7 Statistical Analysis

Statistical analyses comparing both TEO and PAH concentrations before and after slurry phase biotreatment were performed using the SAS System (SAS, 1996). Contaminant concentrations from the EDM-Bio and PAC-Bio soils were compared using the TTEST procedure.

2.4 Results/Discussion

2.4.1 Soil Characteristics

Soil pH, electrical conductivity (EC), total dichloromethane extractable organics (TEO), total organic carbon (TOC) before and after Soxhlet extractions, total

nitrogen (TN), total PAHs, as well as site history characteristics are given in Table 2.3.

In terms of the soil master variables, the textural classes of the contaminated and pristine soils matched well. The soil pH for the EDM and Malmo-C soils matched well, both were slightly alkaline. However, the pH did not compare as well between the PA soils (slightly alkaline) and the Brud-C soil (slightly acidic). The EC values for the contaminated soils were slightly more than three times greater than the respective pristine soils. Total organic carbon and TEO did not compare as well as would have been expected. The loss of organic carbon following TEO extraction did not account for the mass of TEO extracted. The pristine soils as well as the PAL soil had comparable TEO and TOC values among the same soils. The discrepancy between the carbon measurements could be related to the presence of residual solvent or water in the TEO samples, however, all samples were treated in the same manner. Comparisons between the PAC and PAL soils matched well for texture, pH, and EC. Differences in the degree of contamination resulted in greater differences in TEO, TOC, TN and total PAHs.

Table 2.1: Soil and site characteristics.

Soil	EDM	PAC	PAL	Malmo-C	Brud-C	EDM-Bio[+]	PAC-Bio[+]
Location	Edmonton, Alberta	Prince Albert, Saskatchewan	Prince Albert, Saskatchewan	Ellerslie, Alberta	Bruderheim, Alberta	Edmonton, Alberta	Prince Albert, Saskatchewan
Activity[†]	Wood Preserving Creosote	Wood Preserving Creosote	Wood Preserving Creosote	Pristine Site; Research Farm	Pristine Site	Wood Preserving Creosote	Wood Preserving Creosote
Years of Operation[†]	1924 – 1988	1932 – 1972	1932 – 1972	NA	NA	1924 – 1988	1932 – 1972
Sand (%) (> 50 μm)	36.5	94.5	97.2	32.3	97.4	ND	ND
Clay (%)(< 2 μm)	55.2	2.5	1.9	46.6	2.3	ND	ND
Silt (%)(2 - 50 μm)	8.3	3.0	0.9	21.1	0.3	ND	ND
Texture[‡] (hydrometer)	Clay	Sand	Sand	Clay	Sand	ND	ND
pH (H_2O)	7.92	8.29	8.18	8.36	6.88	7.87	8.29
EC (dSm^{-1})	0.461	0.158	0.162	0.141	0.045	0.701	0.158
TOC (%) Before[*]	2.80 ± 0.06	0.97 ± 0.05	0.14 ± 0.01	0.467 ± 0.003	0.0325 ± 0.005	2.38 ± 0.23	0.98 ± 0.01
TOC (%) After[**]	2.56 ± 0.06	0.91 ± 0.05	0.11 ± 0.02	0.378 ± 0.002	0.0265 ± 0.005	2.24 ± 0.24	0.91 ± 0.01
Total N (%)[***]	0.101 ± 0.004	0.026 ± 0.000	0.017 ± 0.001	0.051 ± 0.001	0.011 ± 0.001	0.135 ± 0.008	0.052 ± 0.000
TEO (%)	1.54 ±0.04	0.82 ± 0.02	0.043 ± 0.004	0.016 ± 0.002	0.005 ± 0.003	0.87 ± 0.01	0.83 ± 0.01
Total PAHs' (mg kg^{-1})	1300 ± 70	1500 ± 440	22 ± 5	0.0 ± 0.0	0.0 ± 0.0	83 ± 10	1200 ± 60

[+]Denotes soil after 52 days of biotreatment.
[†]Rutherford et al., 1998.
[‡]Canada Soil Survey Committee, Subcommittee on Soil Classification (1978).
[*,**]before and after soxhlet 16 hrs of Soxhlet extraction.
[*,**,***]All determined on a Carlo Erba Strumentazione NA 1500 Nitrogen/Carbon/Sulfur Analyzer.
'Total PAHs is defined as the sum of 16 PAHs as quantified by GC/MSD.
All percentages are reported on an oven dry soil basis.
ND = Not Determined.

2.4.2.1 Total Dichloromethane Extractable Organics

Total dichloromethane extractable organic (TEO) contents ranged from 1.54 % in the EDM soil to 0.08 % in the PAL soil. A range of contaminated soils, with corresponding pristine soils were combined to produce 100 % contaminated, 50, 25, 12.5, 6.25, 3.13 %, and 100 % pristine treatments (w/w). The treatments were used in the acute toxicity testing (Chapter 3). TEO determinations were conducted over the range of treatment concentrations. The TEO increased linearly with increasing quantities of contaminated soil (Figure 2.1). The R^2 values were all greater than 0.95, indicating a strong linear relationship due to the influence of the point at the highest concentration. Most data points at the lower measured TEO concentrations, for all soils, were slightly less than would be predicted. The slight discrepancy was likely due to the increasing amount of pristine soil, which provided new surfaces for the contaminants to sorb onto. The extractant may have been unable to solubilize all the strongly sorbed contaminants off the soil, resulting in slightly decreased contaminant recovery rates. Interestingly, in the EDM soil treatments, the Malmo-C soil, which had a clay texture and a larger surface area than the sandy textured soils, had the highest quantity of contaminant recovered. Further, the greatest underestimations occurred in the PAC soils with 0.053 and 0.077 % TEO (12.5 and 25 % contamination (w/w). It is unclear why these two treatments deviated from the other values. Perhaps experimental error was the cause of the lower recovery.

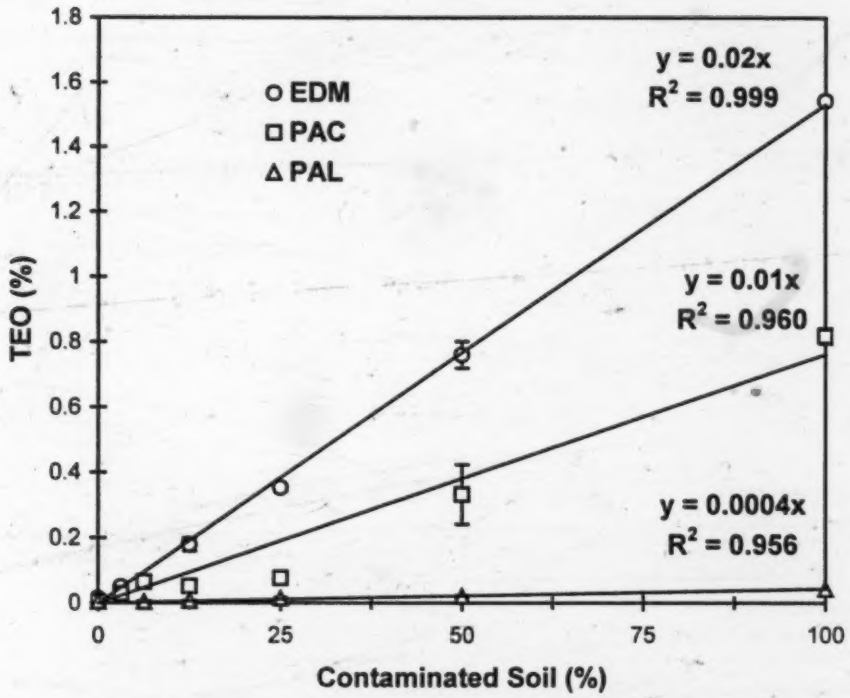

Figure 2.1: TEO concentrations extracted with dichloromethane for aged creosote contaminated soils with increasing quantities of contaminated soil.

2.4.2.2 Biotreatment and TEO

After 52 days of biotreatment the TEO content of the EDM soil was significantly reduced, however the TEO content of the PAC remained unchanged (Table 2.4). In the EDM-Bio soil TEO was reduced by 44 % compared with the original non-biotreated soil. The PAC-Bio soil did not have a significant reduction in TEO concentration. The TEOs in both biotreated soils were still nearly 100 fold greater than the Alberta Tier I criteria of 0.1 % (1000 mg kg^{-1}) after slurry phase biotreatment.

Table 2.4: Summary of TEO Before and After 52 days of slurry phase biotreatment in two weathered creosote contaminated soils. Differences were determined using a TTEST procedure on the SAS system. TEO is reported as the mean value (n = 3).

Soil	TEO (%)	Significant Difference $p < 0.05$
EDM	1.54	Yes
EDM-Bio	0.87	
PAC	0.82	No
PAC-Bio	0.83	
Alberta Tier I Guideline	0.1	

2.4.3 PAH Quantification

The original intent for the addition of the BaP-d_{12} was for use as an internal standard. However, problems with seemingly inconsistent recoveries of BaP-d_{12} forced the use of an external calibration curve using a mixture of PAHs and DBT. Additionally, there may have been unexpected losses of BaP-d_{12} to the soil.

Quality control data for standards and blanks from the individual PAH runs were found to be consistent. That is there were not appreciable quantities of analytes in the blank runs. Further, the PAH standards did not deviate from their expected concentrations. The extra elutions (Section 2.3.6.1) used to determine if any PAHs were retained on the SPE cartridge after the preliminary elutions did not have any detectable concentrations of desired analytes.

2.4.3.1 Soxhlet Extraction Efficiency

The Soxhlet extraction efficiency of the marine sediment (HS-3, NRC) ranged from 17 % in acenapthene to 126 % for benzo[a]pyrene. The overall recovery for total PAHs was 51 %. The broad range of extraction efficiencies (Table 2.5) does not correspond to the high marine sediment (HS-3) PAH recovery rates reported by others using similar extraction and quantification procedures (Zemanek, 1994).

Low recovery rates could be the result of analyte loss through the sample clean up procedure, as well as potential losses to the boiling chips. After Soxhlet extraction, the clean up procedure of the extract conducted by the NRC differed when compared to what was done in this work. The NRC clean up procedure consisted of passing the extract through a column of silica and copper powder, using 20 and 40 % solutions of DCM in diethyl ether. After which, the eluate was transferred to a column of Sephadex LH-20 gel and eluted with a 6:4:3 mixture of cyclohexane, methanol, and DCM. In the clean up procedure used in this thesis, only a LC-Florisil cartridge was used (Section 2.3.6.1). Differences in the two clean up procedures may have partially resulted in the discrepancies seen between the PAHs recovered and the known PAH concentrations.

The lower percent recoveries of the higher molecular weight PAHs, may be due to adsorption onto the SPE packing. The average TEO concentrations for the primary standard (n = 7; 1.67 ± 0.17 %) and the secondary standard (n = 9; 0.98 ± 0.04 %) were consistent amongst each other.

Table 2.5: Soxhlet recoveries of PAHs from the primary NRC standard (HS-3) as determined by GC / MSD. Observed concentrations are reported as the mean (n = 7).

Compound	Molecular Weight ($g\ mol^{-1}$)	NRC ($mg\ kg^{-1}$)	Observed ($mg\ kg^{-1}$)	% Recovery	Range ($mg\ kg^{-1}$) Lower	Upper
Naphthalene	128.2	9	3.0	33.6	1.1	6.2
Acenaphthylene	152.2	0.3	0.2	72.3	0.2	0.2
Acenapthene	154.2	4.5	0.8	16.9	0.2	2.1
Fluorene	166.2	13.3	2.0	15.2	0.6	4.2
Dibenzothiophene	178.2	N/D	2.4	ND	1.5	3.4
Phenanthrene	178.2	85	35.2	41.4	13.3	55.4
Anthracene	184.3	13.4	2.3	17.5	1.3	3.4
Fluoranthene	202.3	60	32.3	53.8	18.7	48.0
Pyrene	202.3	39	24.0	61.4	13.9	34.7
Benz[a]anthracene	228.3	14.6	9.0	61.7	4.1	19.6
Chrysene	228.3	14.1	10.8	76.3	6.6	15.5
Benzo[b]fluoranthene	252.3	7.4	5.8	78.1	3.0	11.2
Benzo[k]fluoranthene	252.3	7.7	6.6	85.1	3.6	10.7
Benzo[a]pyrene	252.3	2.8	3.5	125.6	1.7	6.2
Benzo[ghi]perylene	276.3	5	2.4	48.6	1.1	5.4
Dibenz[a,h]anthracene	276.3	1.3	0.6	43.9	0.2	0.8
Indeno[1,2,3-cd]pyrene	278.4	5.4	2.5	46.7	1.3	5.8
Total PAHs		282.8	143.4	50.7	72.4	232.8

ND = Not determined for the HS-3 standard.

2.4.3.2 Soil PAH Concentrations

As the amount of contaminated soil increased, so did the total PAH concentration (Figures 2.2, 2.3, and 2.4). The total PAHs measured in the 100% (w/w) contaminated soils ranged from 22 mg kg^{-1} in PAL to 1500 mg kg^{-1} in PAC and 1300 mg kg^{-1} the EDM soil (Table 2.6). As with the TEO concentrations (Section 2.4.2.1), a series of soil dilutions were used for the acute toxicity testing (Chapter 3). Thus PAH concentrations were determined for individual soil treatments varying in contaminant concentrations.

Table 2.6a: Individual creosote constituents extracted with dichloromethane for 16 hours in a Soxhlet apparatus and quantified using GC/MSD.

Compound	Molecular Weight ($g\ mol^{-1}$)	PAL ($mg\ kg^{-1}$)	PAC ($mg\ kg^{-1}$)	PAC-Bio ($mg\ kg^{-1}$)	EDM ($mg\ kg^{-1}$)	EDM-Bio ($mg\ kg^{-1}$)
Naphthalene	128.2	0.4	34.0	1.6	3.0	3.1
Acenaphthylene	152.2	ND	ND	0.9	2.4	0.3
Acenapthene	154.2	0.1	118.9	85.8	71.9	1.6
Fluorene	166.2	0.1	114.2	36.5	91.8	2.4
Phenanthrene	178.2	0.0	27.6	2.6	30.0	0.5
Anthracene	178.2	0.4	389.8	73.6	365.3	7.2
Dibenzothiophene	184.3	0.3	165.4	119.3	102.6	15.5
Fluoranthene	202.3	3.4	204.7	233.4	267.1	6.2
Pyrene	202.3	7.1	170.8	192.1	201.0	4.8
Benz[a]anthracene	228.3	1.6	57.7	84.3	51.0	2.0
Chrysene	228.3	2.6	103.1	122.3	84.6	5.1
Benzo[b]fluoranthene	252.3	1.5	36.2	66.0	25.2	12.0
Benzo[k]fluoranthene	252.3	1.8	42.4	53.8	30.6	5.1
Benzo[a]pyrene	252.3	1.7	37.4	62.3	15.6	3.3
Benzo[ghi]perylene	276.3	0.6	8.6	23.0	3.6	6.2
Indeno[1,2,3-cd]pyrene	276.3	0.1	2.5	6.8	1.8	1.4
Dibenz[a,h]anthracene	278.4	0.6	9.2	22.7	5.4	6.6
Total PAHs		22.4	1495.1	1184.3	1322.9	82.7
Reported PAHs		22	1500	1200	1300	83

ND = Not detected

Table 2.6b: Standard deviations for individual creosote constituents extracted with dichloromethane for 16 hours in a Soxhlet apparatus and quantified using GC/MSD.

Compound	Molecular Weight ($g\ mol^{-1}$)	PAL ($mg\ kg^{-1}$)	PAC ($mg\ kg^{-1}$)	PAC-Bio ($mg\ kg^{-1}$)	EDM ($mg\ kg^{-1}$)	EDM-Bio ($mg\ kg^{-1}$)
Naphthalene	128.2	0.1	26.1	0.3	1.0	0.2
Acenaphthylene	152.2	ND	ND	0.2	1.0	0.0
Acenapthene	154.2	0.0	41.4	7.8	8.8	0.0
Fluorene	166.2	0.0	31.6	2.7	6.7	0.2
Phenanthrene	178.2	0.0	8.6	0.2	1.6	0.1
Anthracene	178.2	0.1	95.5	8.0	16.2	0.9
Dibenzothiophene	184.3	0.0	60.8	9.7	10.7	5.0
Fluoranthene	202.3	2.1	50.2	3.8	2.6	0.5
Pyrene	202.3	0.8	43.9	5.7	10.2	0.3
Benz[a]anthracene	228.3	0.2	17.9	2.7	0.5	0.3
Chrysene	228.3	0.3	32.6	6.5	3.1	0.5
Benzo[b]fluoranthene	252.3	0.2	10.8	1.1	1.6	0.7
Benzo[k]fluoranthene	252.3	0.5	11.1	4.9	2.3	0.4
Benzo[a]pyrene	252.3	0.3	12.8	4.6	2.3	0.1
Benzo[ghi]perylene	276.3	0.2	3.5	1.7	0.1	0.3
Indeno[1,2,3-cd]pyrene	276.3	0.0	1.0	1.7	0.0	0.0
Dibenz[a,h]anthracene	278.4	0.2	2.8	1.1	0.1	0.2
Total PAHs		5.1	441.9	62.5	67.4	9.5
Reported		5	440	60	70	10

ND = Not determined

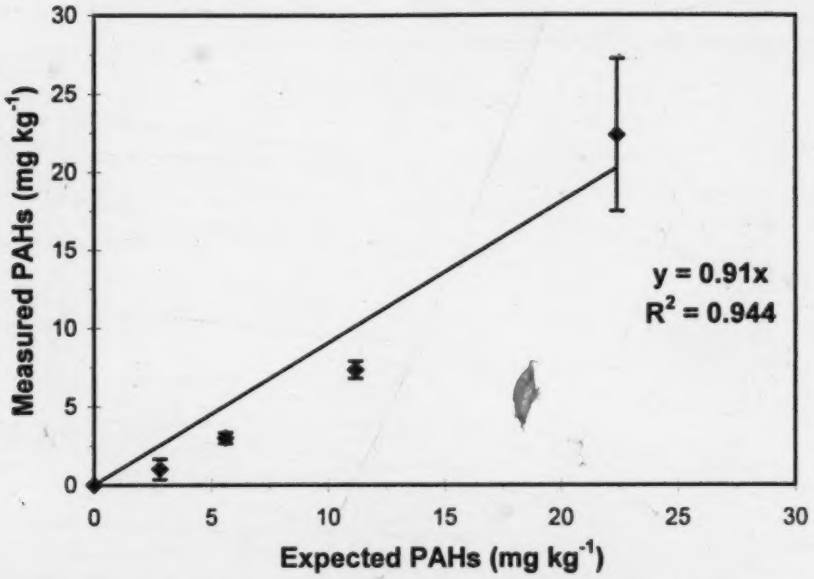

Figure 2.2: Relationship between increasing PAL soil contamination and extractable PAHs.

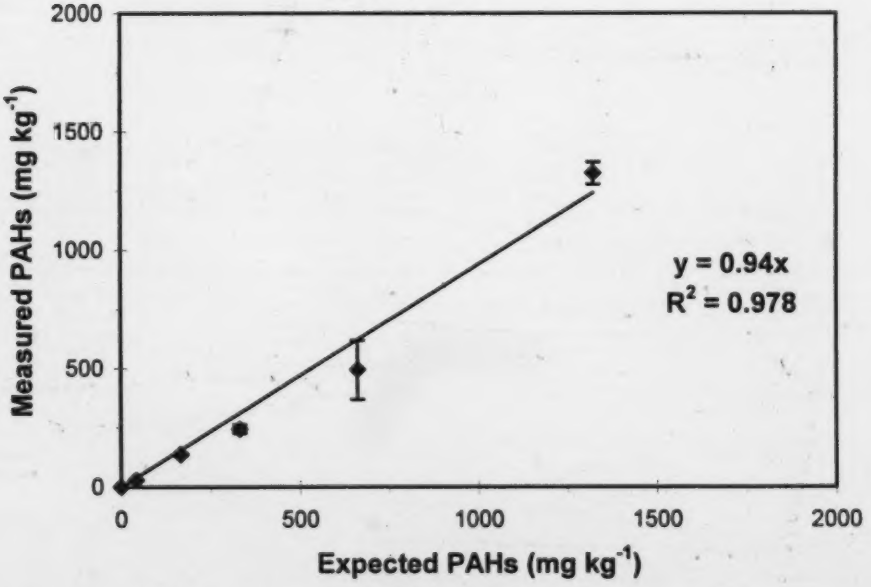

Figure 2.3: Relation between increasing EDM soil contamination and extractable PAHs.

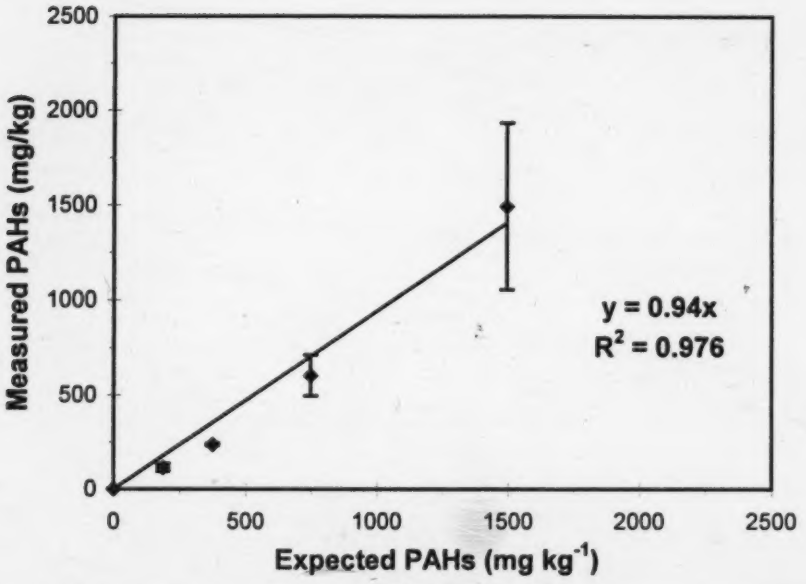

Figure 2.4: Relation between increasing PAC soil contamination and extractable PAHs.

2.4.3.3 PAHs and Biotreatment

Biotreatment has been used as a method to remediate creosote contaminated soils (Ellis et al., 1991; Rutherford et al., 1998). With increasing time, the lower molecular weight PAHs are degraded in a relatively short time, compared to PAHs with five or more aromatic rings.

The 52 days of biotreatment for the PAC and EDM soils resulted in a decrease of PAHs from the soil (Table 2.7; Figure 2.5 and 2.6). The EDM soil had a greater response to the biotreatment with both the TEO and PAHs decreasing more than in the PAC soil. In the PACBio treatment, there were only reductions for individual PAHs that had three or less aromatic rings. For those PAHs the reduction after treatment ranged from greater than 94 % for naphthalene to 27 % for acenapthene and dibenzothiophene. PAHs with more than three rings did not show a significant decrease in concentration after the biotreatment of the PAC soil.

In the EDM-Bio soil, there were decreases in nearly all individual PAHs that were quantified. Naphthalene, benzo(ghi)perylene and dibenz[a,h]anthracene were

the only PAHs that did not show a decrease in concentration after treatment. Soil naphthalene losses may have occurred during soil handling because of naphthalene volatility. The losses before biotreatment may be the reason naphthalene did not show statistically significant reductions after biotreatment. All quantified three ringed PAHs had a reduction of greater than 85 %. The four ringed compounds in the EDM soil were all reduced by greater than 94 %, which is a sharp contrast from the PAC soil. There were some reductions in PAHs in the EDM soil that have five rings, but they were not as dramatic as the compounds that had four rings or less. The only six ring PAH which had a reduction was indeno[1,2,3-cd]pyrene.

Table 2.7: Summary of quantified creosote constituents before and after 52 days of slurry phase biotreatment. Values are reported as means (n = 3). Soils were compared using a TTEST procedure. Compounds are listed in their order of elution.

	Soil Concentration (mg kg^{-1})					Significant
Compound	EDM	EDM-Bio	Significant	PAC	PAC-Bio	$p < 0.05$
Naphthalene [2]	3	3	No	34	2	No
Acenaphthylene [3]	2	0	Yes†	ND	1	-
Acenapthene [3]	72	2	Yes	119	86	No
Fluorene [3]	92	2	Yes	114	36	Yes‡
Dibenzothiophene [3]	103	15	Yes	165	119	Yes
Phenanthrene [3]	30	1	Yes	28	3	Yes
Anthracene [3]	365	7	Yes	390	74	No
Fluoranthene [4]	267	6	Yes	205	233	No
Pyrene [4]	201	5	Yes	171	192	No
Benz[a]anthracene [4]	51	2	Yes	58	84	No
Chrysene [4]	85	5	Yes	103	122	No
Benzo[b]fluoranthene [5]	25	12	Yes	36	66	Yes
Benzo[k]fluoranthene [5]	31	5	Yes	42	54	No
Benzo[a]pyrene [5]	16	3	Yes	37	62	Yes
Benzo[ghi]perylene [6]	4	6	Yes	9	23	Yes
Indeno[1,2,3-cd]pyrene [6]	2	1	Yes	2	7	Yes
Dibenz[a,h]anthracene [5]	5	7	Yes	9	23	Yes
Total Constituents	1354	82	Yes	1522	1187	No

[ ] = number of rings
†Significant at $p < 0.0729$
‡Significant at $p < 0.0504$
ND = Not detected

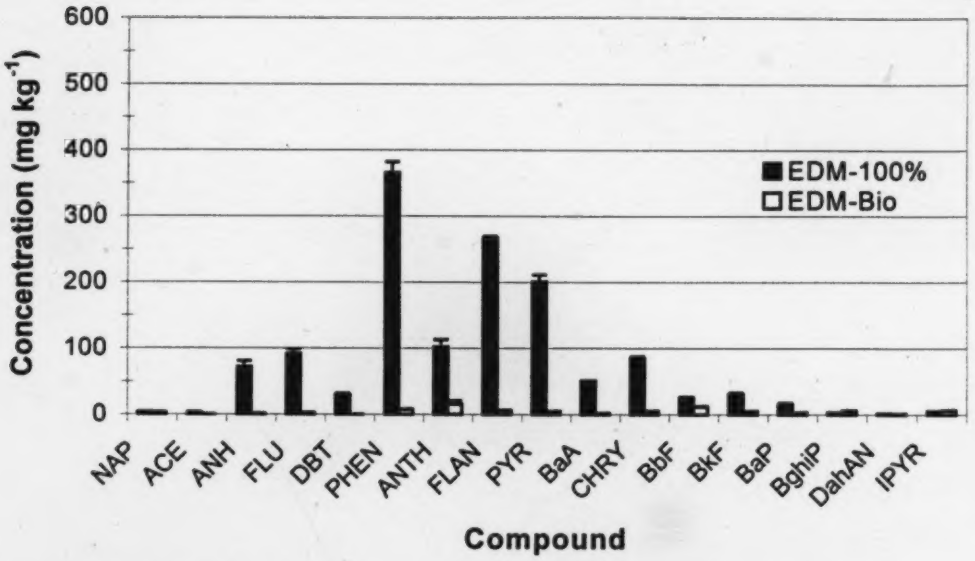

Figure 2.5: Total PAH concentrations (mg kg^{-1}) for the EDM soil before and after 52 days of slurry phase biotreatment at 22°C, with continual rotation and under dark conditions.

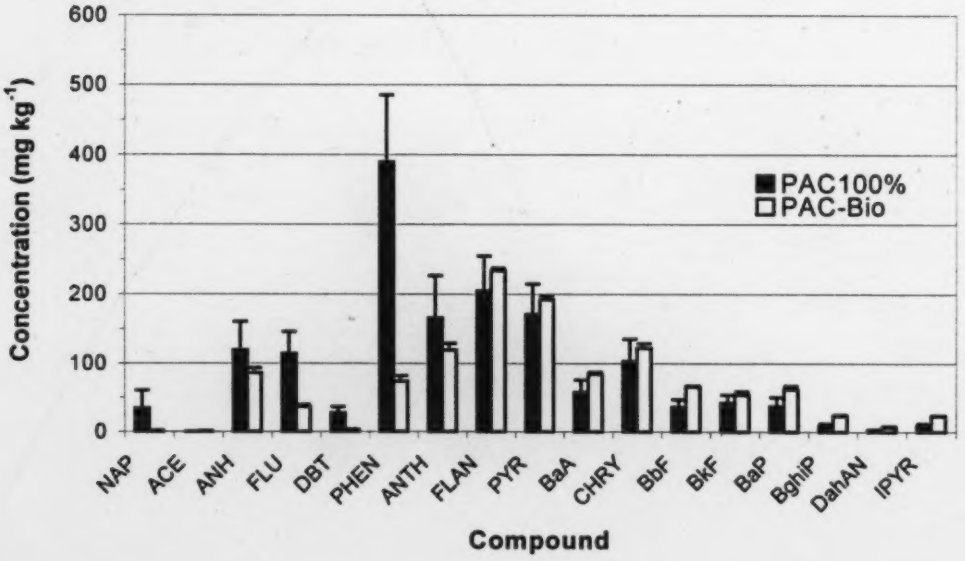

Figure 2.6: Total PAH concentrations (mg kg^{-1}) for the PAC soil before and after 52 days of slurry phase biotreatment at 22°C, with continual rotation and under dark conditions.

2.5 Conclusions

Overall the soil master variables in the pristine soils matched well to their respective aged creosote contaminated soils, especially in terms of textural classes. The other master variables matched to varying degrees, the greatest discrepancy was between the TEO and TOC concentrations in the contaminated and biotreated soils.

Contaminant concentrations in the 100 % contaminated soils ranged in TEO from 0.043 ± 0.004 to 1.54 ± 0.04 mg $kg^{-1}$and total PAH concentrations ranged from 22 ± 5 to 1500 ± 440 mg kg^{-1}. The TEO and PAH concentrations increased linearly with increasing concentrations of contaminated soil. The TEO concentrations in the PAC soil reflect the strong influence of the 100 % contaminated soil value. In the lower contaminant concentrations, the linear relationship appeared to be weaker, however the higher concentration value appeared to control the fit of the trendline.

Total PAHs decreased significantly in the EDM soil after 52 days of slurry phase biotreatment. The PAC-Bio soil did not have the same reduction of creosote constituents. Soil habitat for microbes as well as surface area for sorption of contaminants, may be influencing degradation and availability of contaminants to microbes.

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Chapter 3: Acute Ecotoxicity Testing

3.1 Introduction

Ecotoxicology, an extension of classical toxicology, attempts to study the fate and toxicity of xenobiotics in an ecosystem (Kendall et al., 1996). Furthermore, ecotoxicology integrates several fields of study such as ecology, chemistry, biology and soil science. To understand and protect the health of soils, specifically following contaminated site remediation, requires a progression beyond single discipline research to an integrated ecotoxicological approach.

Within an ecotoxicological approach there must be a mechanism to evaluate the risk imposed by exposure to contaminants. As used here, risk refers to the probability of an adverse outcome (Faustman and Omenn, 1996) within a specified time period. For a risk to occur, three elements must be present: a hazard, a pathway, and a receptor (Figure 3.1). If one element is missing, then there can not be a risk. In the context of this thesis, the hazards are the constituents of creosote. Coal tar creosote is a complex mixture of several hundred identifiable chemical components. The principal constituent classes found in creosote are: the polycyclic aromatic hydrocarbons (PAHs); phenolic compounds; and N-, O- and S- heterocyclics (Mueller et al., 1989). Acute creosote toxicity is well documented, and the ecotoxicological ramifications of environmental exposures to PAHs, the major constituent class of creosote (Mueller et al., 1989), are detailed in the literature for several different ecotoxicological approaches. Studies that combine several biological indicators such as microbes, plants, and earthworms, have been used to assess the toxicity of various PAHs (Aprill et al., 1990; Hund and Traunspurger, 1994; Kelsey and Alexander, 1997; and Salanitro et al., 1997). Typically, the major pathways of exposure examined in an ecotoxiclogical study of PAHs in the soil are ingestion and/or dermal contact. For this thesis the receptor being studied was the earthworm *Eisenia fetida*.

Figure 3.1: Elements of risk (modified from CCME, 1996).

The soil connects many global cycles and can over time attenuate organic chemical contaminants that are released into the environment, which potentially reduces the bioavailability of a contaminant (Hatzinger and Alexander, 1995; Chung and Alexander, 1998). However, the accumulation of xenobiotic compounds in the soil can have adverse consequences for soil organisms. As toxic chemical concentrations increase in the soil, soil organism survival, biomass, and respiration can decline (Simini et al., 1995; Martikainen, 1996). Ecotoxicological research requires suitable indicator species with which to make quantitative measurements of soil health. Earthworms can function as such indicators of soil health. For several years, earthworms have been used to study the environmental impacts of chemicals such as pesticides, herbicides, metals, and polycyclic aromatic hydrocarbons (PAHs) in the soil (Edwards and Bohlen, 1996). Earthworms are: large, numerous and easily sampled; widely distributed geographically; in full contact with their substrate and ingest large volumes of it; and able to respond to chemicals not only through mortality but also through behavioral, reproductive and growth rate changes (Edwards and Bohlen, 1996). Thus, earthworms possess key characteristics necessary for environmental monitoring.

Soil organisms, such as earthworms, are able to integrate directly the interactions between the contaminant and the soil matrix. Responses measured in bioassays, to

the biologically available component of a contaminant mixture, provide a direct evaluation of the environmentally relevant toxicity to compare with chemical analysis (Keddy et al., 1995). Therefore, it is bioavailability that relates to the risk of a toxicant. Bioavailability, as used here, refers to the internal dose of a xenobiotic chemical, which exists in the soil, enters the organism, and initiates a measurable biological response. Figure 3.2 illustrates the three principal modifiers of bioavailability: contaminant properties, the soil matrix, and the organism. Chemicals that are not bioavailable do not present a risk to organisms, in terms of toxicity, they may, however make the soil non-habitable if for example they cause soil to become hydrophobic and remain too dry for survival of soil organisms. Earthworm species have adapted for many different terrestrial habitats, however, they can not survive in dry soils (Sims and Gerard, 1985).

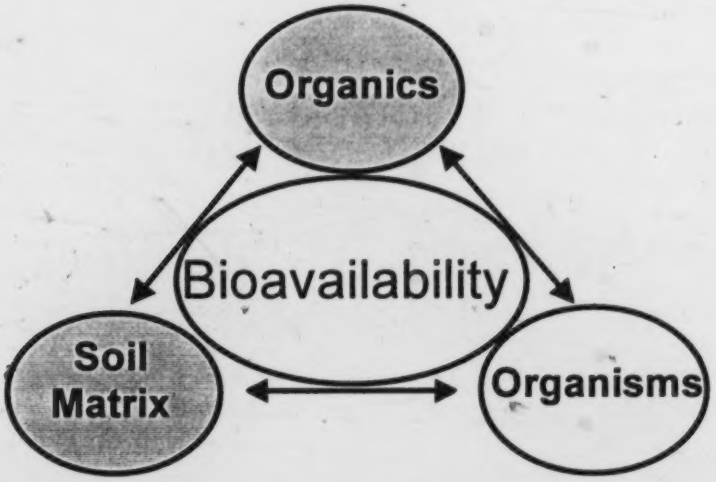

Figure 3.2: Principle modifiers of bioavailability (modified from Tiedje, 1993).

Alberta Tier I guidelines currently focus on total chemical concentration of contaminants in the soil. Regulations limited to solvent-extractable concentrations may be assigning inappropriate levels of risk to organic contaminants in the soil (Hund and Traunspurger, 1994; Loehr and Webster, 1996). Several criticisms exist regarding the strict reliance on solvent-extraction data to predict environmental risks. Lambolez et al. (1994) cite the difficulties in identifying certain micropollutants

within a complex waste mixture and predicting synergistic or antagonistic interactions between pollutants as reasons why toxicological studies need to complement chemical analysis. Site history can dictate which classes of compounds will be screened for in a site assessment. Failure to quantify all initial compounds and degradation metabolites will limit the usefulness of regulations based on chemical extractability (Hund and Traunspurger, 1994). Furthermore, strict reliance on chemical data without complementary biological testing could compromise the value of regulatory guidelines and remediation objectives.

3.2 Objective

The objectives of the work reported in this chapter were twofold. Firstly, to determine if slurry phase biotreatment of weathered creosote contaminated soils would reduce acute toxicity in *Eisenia fetida* compared to the toxicity of soils that did not undergo a bioteatment process. Secondly, to determine if toxicity could be predicted using chemical data. Chemical predictions could be tested against the results of biological observations. For the purposes of this thesis acute toxicity refers to exposure periods of earthworms, which are not greater than fourteen days. Toxicity was measured using a fourteen day earthworm survival assay.

3.3 Materials and Methods

3.3.1 Soils

Three core contaminated soils (CCS) from two weathered creosote sites were evaluated for their acute toxicity. Soils used in the acute toxicity testing are characterized in detail in Chapter 2. The first CCS had a clay texture and was from an industrial site in Edmonton, Alberta (EDM) which contained admixed surface and subsurface material. The second CCS was sampled from the subsurface at a site in Prince Albert, Saskatchewan (PAC) and had a sandy texture. The final CCS (PAL) was from the same site at Prince Albert, however, it was sampled from a different part of the site and had lower concentrations of total extractable organics (TEO) and total PAHs compared to PAC. The contaminated core soils were chosen to reflect contrasting soil and contaminant characteristics (Table 2.3). Use of the term

contaminated soil, in this thesis, refers to an admixture of contaminated soil horizons and PGM.

At the beginning of the acute toxicity experiments (spring 1997) the test soils were removed from the cooler (4°C) and allowed to air dry at 22 °C, in fume hoods. The pristine control soils were collected in 1997 and processed as described in Section 2.3.1.2.

Soil bulk densities for the clay textured soils (EDM and Malmo-C) were higher than expected. A typical bulk density value for a clay soil is approximately 1.05 g cm^{-3} (Hausenbuiller, 1985). The EDM and Malmo-C soils were both powdery with limited aggregation; soils with such characteristics would likely have higher bulk densities than would be predicted by texture alone. The presence of enriched carbonates in the PA soils as well as the Malmo-C soil resulted in an elevated soil pH. The effervescence of the carbonate enriched soils after addition of 10 % (v/v) HCl confirmed the presence of carbonates.

Table 3.1: Contaminated and pristine soil characteristics used in earthworm acute toxicity assays*.

Soil	Location	Activity†	Years of Operation†	Texture‡ (hydrometer)	Bulk Density+ (g cm^{-3})	EC (ds m^{-1})	pH (H_2O)
EDM	Edmonton, Alberta	Wood Preserving	1924 – 1988	Clay	1.31 ± 0.01	0.461	7.92
PAC	Prince Albert, Saskatchewan	Wood Preserving	1932 – 1972	Sand	1.53 ± 0.01	0.158	8.29
PAL	Prince Albert, Saskatchewan	Wood Preserving	1932 – 1972	Sand	1.44 ± 0.02	0.162	8.18
Malmo-C	Ellerslie, Alberta	Research Farm	NA	Clay	1.33 ± 0.01	0.141	8.36
Brud-C	Bruderheim, Alberta	Pristine Site	NA	Sand	1.50 ± 0.01	0.045	6.88

*Detailed descriptions of methods used to measure the reported soil characteristics are provided in Chapter 2.
+Based on air-dried soils, see Section 3.3.5.2.
†Rutherford et al., 1998.
‡Canada Soil Survey Committee, Subcommittee on Soil Classification (1978).

3.3.2 Slurry Phase Biotreatment

Two weathered creosote contaminated soils (EDM and PAC) were subjected to 52 days of slurry phase biotreatment. Details on the biotreatment protocol are detailed in Section 2.3.3.

3.3.3 Earthworms

For the purpose of this thesis, the terms worm and earthworm refer to *Eisenia fetida*. *E. fetida* from the Carolina Biological Supply Company (Burlington, NC) were used for all acute toxicity tests except for the PAL acute toxicity assay, which used *E. fetida* from a local supplier (Dirt Willy Farm, Ardrossan, AB). The earthworms used were mature, with clitellum. Overall the mean individual earthworm mass was 389 ± 97.2 mg and ranged in mass from > 160 mg to < 713 mg. The mean masses for each toxicity study were: EDM = 336 ± 100.0 mg, PAL = 391 ± 86.5 mg and PAC = 436 ± 77.8 mg. The worms, when not in toxicity assays, were maintained in 15.5 L (40 X 32 X 12 cm) translucent, covered, plastic containers (Rubber Maid) with 1/4 inch (6.35 mm) holes drilled in the lid to allow for air exchange. The soil medium for culturing the worms before starting any toxicity testing was the medium in which the worms were packaged and shipped (Magic Worm Bedding; Carolina Biological Supply Co.). The medium was replaced with fresh Magic Worm Bedding after 3 months of use. Several hundred worms were kept in each container at any one time.

The containers were stored in a growth chamber at 22 ± 2°C under constant light to encourage burrowing. The growth chamber provided a favorable environment for worms, prior to toxicity testing. The average illuminance using a 1 second integration time was 4000 ± 200 lux in the growth chamber; on the surface of the soil with the container lid closed the illuminance was 3200 ± 190 lux (LI-188 (LI-COR) Integrating Quantum / Radiometer / Photometer).

The worm diet consisted of a total of 40 - 50 g (dry weight) of food, 20 - 25 g each of oat bran and corn meal. Equal masses of oat bran and corn meal were weighed out, mixed together and then incorporated into the soil. The worms were fed

40 – 50 g of meal every 7 - 10 days. The soil was kept moist by adding double deionized water (DDW) (Milli-Q™ Water System) to the worm bedding every 7 - 10 days, as required. No specific moisture content was set for the worm bedding, just the maintenance of a moist soil without free water remaining on the surface. *E. fetida* habitats typically occur under damp rotting vegetation and low pH (4.3 – 7.5) conditions in organic soils and compost piles (Sims and Gerard, 1985). Environmental conditions were selected to approximate the natural habitat of *E. fetida.*

3.3.4 Chemical Characterization of Creosote Constituents - *Summary*

Information on the chemical characterization of creosote contaminated soils is detailed in Chapter 2 and summarized in Table 3.2. Total extractable organic contents for all core contaminated (100 %) and biotreated soils exceeded Alberta Tier I guidelines except for the PAL soil (Table 3.2). Total PAH concentrations exceeded regulated values for all core contaminated (100 %) and biotreated soils. The EDM-Bio soil showed a greater response to 52 days of biotreatment, in terms of concentration reductions, for both the TEO and total PAHs than did the PAC-Bio soil. Only the total PAH concentration decreased in the PAC soil after biotreatment. The PAC-Bio total extractable organic content was not statistically different, after 52 days, from the pretreatment soil.

Table 3.2: Summary and comparison of contaminant characteristics from the core creosote contaminated and biotreated soils to Alberta Tier I guidelines.

Soil	Total PAHs* ($mg\ kg^{-1}$)	TEO (%)	PAH/TEO
EDM	1320 ± 50	1.54 ± 0.04	0.086
EDM-Bio+	80 ± 10	0.87 ± 0.01	0.0092
PAC	1500 ± 440	0.82 ± 0.02	0.18
PAC-Bio+	1180 ± 50	0.83 ± 0.01	0.14
PAL	20 ± 5	0.043 ± 0.004	0.047
Alberta Tier I	1.0	0.1	0.001‡

*Note total PAHs is defined as the sum of 16 PAHs as quantified by GC / MSD.
+Denotes soil after 52 days of biotreatment.
‡This parameter is not actually part of the Alberta Tier I guidelines.

3.3.5 Acute Toxicity Testing (14 day earthworm survival)

Several protocols for the acute earthworm toxicity test have been proposed (ASTM, 1995; Greene et al., 1989; OECD, 1984). The earthworm toxicity bioassay used here incorporates many of the principles described in the previously cited works, and is described in detail below.

3.3.5.1 Experimental Design

A total of eight soil treatments were used to test for the acute toxicity of EDM and PAC soil samples. Seven treatments were dilutions of the 100 % contaminated core soils: 100, 50, 25, 12.5, 6.25, and 3.13 % (w/w) with a corresponding mass of pristine soil; including the seventh treatment, 100 % pristine soil, which was the control. The Malmo-C soil was the control used in the EDM toxicity tests and Brud-C was the control for the PA soil treatments. The eighth treatment consisted of either the EDM or PAC (100 %) soil after undergoing 52 days of slurry phase biotreatment (EDM-Bio or PAC-Bio) as described in Section 2.3.3. The seven soil dilutions were used in the acute toxicity testing of PAL soil samples, however PAL soil did not undergo any biotreatment. All soil treatments were replicated three times except for the biotreated soils, which had no replication in the acute toxicity tests because of limited quantities of available soil.

Earthworm incubation vessels consisted of 1-L, wide mouth mason jars (Bernardin) with Snap Lids. The lids for all the jars had two 1/4 inch (6.35 mm) holes drilled into the top, allowing for air exchange. Filter paper (Whatman Qualitative No. 1, 9 cm diameter) was placed underneath each lid to reduce water evaporation during the toxicity tests. Before use, the jars were annealed at 560°C (Department of Chemistry) overnight to remove strain in the glass and prevent cracking or breaking during the biotreatment process, while rotating on the rollerbottle apparatus.

3.3.5.2 Soil Preparation

Preparation of the soils for the toxicity test involved the calculation of the appropriate gravimetric moisture content at which to incubate the soils. First,

measurements of initial soil moisture contents (air-dried), and bulk densities under conditions of the toxicity test were conducted for all soils used. The initial moisture content was determined by adding 30-50 g of air-dried soil to a pre-weighed aluminum container. Soil containers were placed in a drying oven at 105°C for 24 hours. The soils were then removed from the oven and allowed to cool to room temperature in a glass desiccator containing a Silica Gel desiccant (Reagent grade, 6-16 Mesh, Fisher Scientific). The soils were placed back in the oven and weighed again after 48 hours to determine if there was any appreciable change in the mass. When there was no appreciable change in mass, the soils were considered to be oven-dry. Once cool, the tins were weighed and the moisture content (θ_m) was calculated (Topp, 1993; Equation 3.1). Each soil used was analyzed in triplicate.

$$\theta_m = \frac{Mass\ of\ Moist\ Soil - Mass\ of\ Oven\ Dried\ Soil}{Mass\ of\ Oven\ Dried\ Soil} * 100\% \qquad \text{(Equation 3.1)}$$

Bulk density was determined using homogenized air-dried soils that were previously passed through a 2 mm sieve. The soils were processed in the same manner as all the other soils used in the toxicity experiments (Section 2.3.1.1). The soil was weighed into a 250 ml graduated cylinder. The cylinder was dropped 5 times on the bench top from a height of approximately 5 cm. The soil in the cylinder was then slightly shaken to level the surface; the volume and mass of soil were recorded, and the soil bulk density (ρ_b) was calculated (Equation 3.2).

$$\rho_b = \left({SoilMass(g)} \Big/ {Volume(ml)} \right) \qquad \text{(Equation 3.2)}$$

Once the air-dried moisture contents were determined, soil treatments were initiated by adding 300 g (oven dry basis) of each air-dried soil or combination of contaminated and control pristine soils to the earthworm incubation vessels. The contaminated and control pristine soils were homogenized by placing each vessel horizontally on a rollerbottle apparatus. The rollerbottle apparatus continuously rotated the jars (3.7 rpm) in a dark room for 20 hours at 22°C. After homogenization, each jar had 15 - 25 g of soil removed to determine an adjusted moisture content (Equation 3.1; Topp, 1993). The new homogenized soil moisture content was used to

calculate the required mass of water necessary to bring each soil to 60 % Water Filled Pore Space (WFP) (Equation 3.3) (Linn and Doran, 1984).

$$WFP = \left(\theta_v / TP\right) * (100\%) \qquad \text{(Equation 3.3)}$$

Where:

$$\theta_v = (\theta_m) * (\rho_b) \qquad \text{(Equation 3.4)}$$

$$TP = \left(1 - \rho_b / \rho_p\right) * (100) \qquad \text{TP = Total Porosity} \qquad \text{(Equation 3.5)}$$

soil particle density (ρ_p) = 2.65 g cm^{-3} (Hausenbuiller, 1985)

The calculated mass of water was determined by rearrangement of Equations 3.3, 3.4 and 3.5. Double deionized water (DDW) (Milli-Q™ Water System) was applied to the homogenized soil surface in the vessels by using a spray bottle. Applying water as a fine mist prevented clumping which occurred in the soils with a high clay content (EDM and Malmo) when compared to adding water directly (no misting). The jars were left overnight, undisturbed, with the lids on to allow infiltration of water into the soil pores. The next day the soils were mixed using a metal soil spatula to distribute evenly any remaining dry soil into the moist soil. The soil was then ready for the addition of the earthworms.

3.3.5.3 Earthworm Additions

The earthworms were individually weighed and counted, then placed within the incubation vessels. Each vessel received 10 worms placed on the surface of the soil. The jars were arranged randomly and kept in a fumehood under constant temperature (22 ± 1°C) and lighted conditions (800 ± 120 lux). The worms were observed a few hours after their introduction to the soil to see how many had initially burrowed into the substrate. No effort was made to force the worms to burrow into the soil. Over the next 14 days the worms were monitored daily to determine their survival within each treatment vessel. In accordance with standard protocols, the acute toxicity test formally ended after 14 days. In some cases the worms which

survived 14 days were exposed for longer times, but this was not standard throughout all treatments. These extended assays aimed to gain a better understanding of earthworm responses to subchronic exposures. No food was provided to any worms during the toxicity tests, regardless of duration.

To monitor survival, the worms were emptied out of the jars and counted daily. Soil was replaced into the corresponding jar and the live worms were placed back on the surface. Any dead worms were removed from the test jars, according to standard protocols. A worm was considered dead if it did not respond to a touching stimulus. As well, the soil + jar + worm weight was monitored and kept constant by adding water (by misting), as required to the surface of the soil. Additional water was added to maintain a constant moisture content of 60 % WFP.

3.3.6 Statistical Analysis

Statistical analyses of earthworm data were performed using the SAS System (SAS, 1996). Calculated maximum earthworm days and concentrations lethal to 50 % of the population (LC_{50}) were derived using a nonlinear least squares procedure.

3.4 Results and Discussion

3.4.1 60% Water Filled Pore Space

Some standard bioassays use a water content based on adding water as a percentage of the mass of soil added (OECD, 1984). Other bioassays use a percentage of water holding capacity as the desired level of hydration (ASTM, 1995; Greene et al., 1989). Soils that differ in texture will have a different balance between air and water filled pores, if water is added based on a percentage of soil mass. The 60 % WFP level was chosen as the moisture content at which to incubate the earthworms during the toxicity tests because, as Linn and Doran (1984) point out; WFP is a simple and reliable parameter. Using water filled pore space for determining moisture conditions allows for direct comparisons of biological activity between soils with differing bulk densities or water contents. It also maintains a constant ratio of air:water filled pores regardless of texture. Further, maximum

aerobic microbial activity occurs at 60 % WFP, as measured by CO_2 production and O_2 uptake (Linn and Doran, 1984). Finally, soils that are contaminated with oily constituents may have altered soil-water relations due to the presence of hydrophobic residues. Water filled pore space was chosen as the criterion for soil moisture because it would not be influenced by oily contaminants and it would provide a constant soil aeration and water balance in soils that differed in texture.

3.4.2 Acute Toxicity Testing

3.4.2.1 EDM Acute Toxicity Test

Eisenia fetida survival was 100 % in the Malmo pristine (control) soil (Figure 3.3). As the amount of contaminated soil increased there was a corresponding decrease in the survival times of the earthworms. In the EDM 100 % (TEO = 1.54 %) contaminated soil all worms died within 2 days, while in the 3.13 % (TEO = 0.050 %) treatment, they all died within 7 days. Although the EDM-Bio soil exceeded Alberta Tier I criteria by as much as 80 fold for total PAHs and by as much as 8.7 fold for TEO, all worms survived for the entire acute toxicity testing period. Moreover, worms in the EDM biotreated soil were still alive at day 51 when the experiment was terminated. This suggests that biotreatment removed the acute toxicity associated with EDM 100 %. The toxicity test was stopped at day 51 so that the worms could be archived and stored in liquid nitrogen for a subsequent chronic toxicity study (Chapter 4).

Earthworm activity decreased with increasing concentration and time of exposure to the contaminated soils. Worms did not burrow into the 100, 50, 25, 12.5 and 6.25 % (w/w) treatments but were active under the surface in the 3.13 % contaminated, 100 % pristine, and the biotreated soils (w/w). Earthworms that did burrow were active as evident by channels that were observed within the soil during daily worm monitoring. Additionally, subsurface worms were active and responsive to touch during the daily inspection of each soil. Burrowing worms in the contaminated soils displayed decreasing activity with increasing time of exposure. Worms that did not burrow displayed limited to no response when probed.

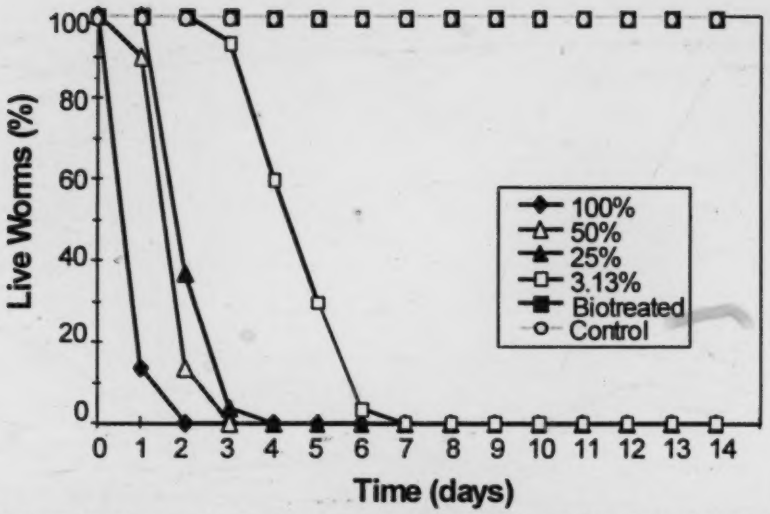

Figure 3.3: *E. fetida* survival after exposure to EDM soil dilutions for 14 days at 22 ± 1°C under continuously lighted conditions. Ten worms per vessel were initially placed on the surface of each soil treatment. Each treatment was replicated three times except for the biotreated soil, which had no replication because of limited quantities of available soil. The 6.25 and 12.5 % treatments follow a similar trend to the other soil dilutions, data not presented.

3.4.2.2 PAL Acute Toxicity Test

Eisenia fetida survival averaged 97 % in the pristine Bruderheim (control) soil (Figure 3.4). There was one earthworm death in a control treatment on day 3 of the toxicity test. This death might be related to the stress of handling the worms in preparation for the toxicity test. The worm that died did not burrow into the soil after day one and thus may have been injured or unhealthy from the start. All other control worms burrowed into the soil and were active within the soil throughout the entire test period. In contrast to the pristine soil, all worms in all contaminated soil dilutions survived for 14 days.

The PAL soil does not appear to be acutely toxic because there was 100 % survival of all worms exposed to contaminated soils for the first 18 days of the toxicity test. After day 18, mortality of worms was observed in the 100 % (TEO = 0.043 %) contaminated soil until day 32, when mortality ceased at 53 % survival. After 41 days of exposure, 50 % of the worms were still alive in the PAL 100 % soil.

The experiment was terminated after day 41 because there did not seem to be any unusual activity and survival rates were plateauing.

In the contaminated soil dilutions 3.13 % and 6.25 % (w/w) , there was 100 % survival for the 41 days of the experiment. In all other treatments, except the 100 % contaminated soil there was 97 % survival for the entire experiment.

Earthworm behavior also suggested the soil was not acutely toxic. Worms in the PAL toxicity test were often found actively entangled in earthworm-balls and were mobile under the surface. Some worms were also engaged in reproductive behavior. Copulating worms lie head to tail with the clitellar region of one worm tightly attached to the spermathecal region of the other worm (Sims and Gerard, 1985). Earthworm behavior was interpreted to be consistent with healthy worms engaging in regular behavior patterns. After 10 days in the PAL 100 % treatment, some of the worms would periodically be found remaining on the surface after being placed on top of the soils. In all other treatments, the worms were always under the surface for the entire 41 day duration of the experiment.

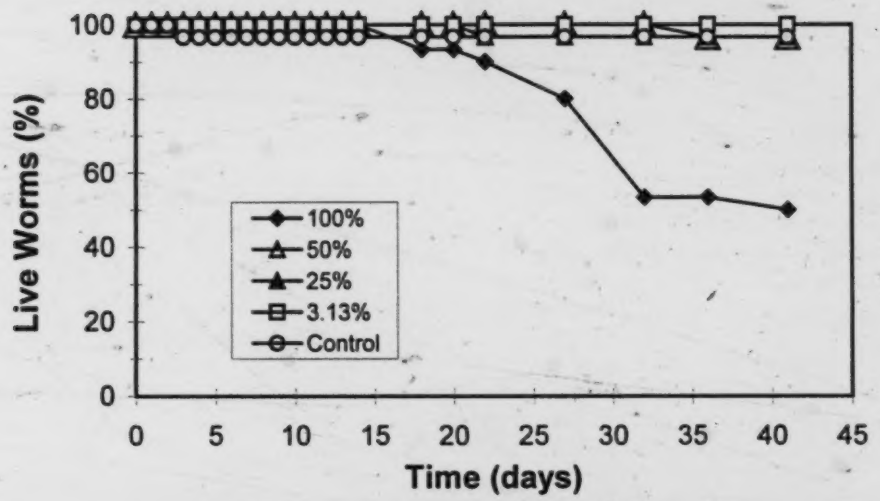

Figure 3.4: *E. fetida* survival after exposure to PAL soil dilutions for 41 days at 22 ± 1°C under continuously lighted conditions. Ten worms per vessel were initially placed on the surface of each soil treatment. Each treatment was replicated three times. The 6.25 and 12.5 % treatments follow a similar trend to the other soil dilutions, data not presented.

3.4.2.3 PAC Acute Toxicity Test

The PAC toxicity assay results were a strong contrast to the EDM and PAL acute toxicity assays. There was 100 % survival in the Bruderheim control soil (Figure 3.5). As the amount of contaminated soil increased in each treatment there was a corresponding decrease in the earthworm survival time. All worms exposed to any amount of contaminated soil were dead by day 4. All worms died within one day in the PAC 100 % (TEO = 0.82 %) soil and within two days in the PAC-Bio soil, suggesting that both were acutely toxic and the PAC-Bio soil was only marginally less so than the PAC soil. The PAC 100 % treatment exhibited greater acute toxicity than the EDM or PAL soils of the same dilution because the earthworms survived for less than one day in the PAC soil. Furthermore, in the 3.13 % dilution (TEO = 0.029 %), the worms survived for 4 days, as compared to 7 days in the EDM 3.13 % treatment and 41 days in the PAL 3.13 % treatment.

In the PAC 100 % treatment there was little to no earthworm movement, even after a few hours of exposure to the creosote contaminated soil. In contrast, worms in

the control soil were active and displayed no physical signs of acute toxicity, even after 14 days of exposure. Over time, worms exposed to contaminated soil treatments displayed limited to no response when probed by touch. As the amount of contamination increased, the earthworms were less likely to burrow into the soil, even under lighted conditions. Earthworms react to lighted conditions (Edwards and Bohlen, 1996) and when exposed to strong light respond by the "withdrawal reflex" (Lee, 1985). A preference for the worms to remain exposed to lighted conditions rather than burrowing into contaminated soil implies a type of sensitivity to the chemicals within the soil. Acute toxicity may have resulted from toxic lower molecular weight volatile and/or water soluble compounds such as naphthalene and/or a multitude of phenolic compounds, which can occur in creosote mixtures. The toxicity of chlorophenols to earthworms such as *E. fetida* correlate to the soil solution concentration and thus, using adsorption coefficients, bioavailability can be predicted (van Gestel and Ma, 1988). Additionally, other lower molecular weight compounds may have been dermally irritating to earthworms, resulting in a lack of burrowing activity after exposure to contaminated soil. Edwards and Bohlen (1996) comment that a wide range of chemicals, such as acids, pesticides and detergents, behave as irritants which will cause earthworms to surface.

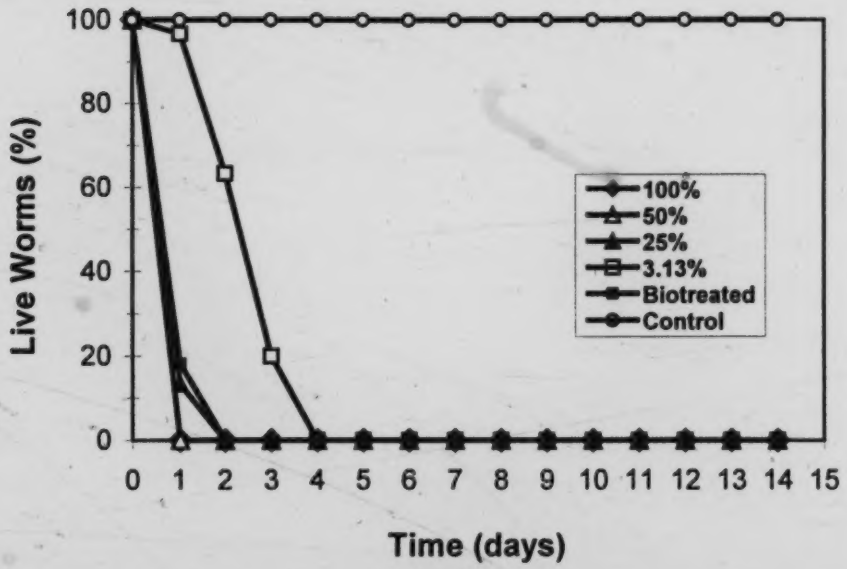

Figure 3.5: *E. fetida* survival after exposure to PAC soil dilutions for 14 days at 22 ± 1°C under continuously lighted conditions. Ten worms per vessel were initially placed on the surface of each soil treatment. Each treatment was replicated three times except for the biotreated soil, which had no replication because of limited quantities of available soil. The 6.25 and 12.5 % treatments follow a similar trend to the other soil dilutions, data not presented.

3.4.3 Earthworm-days

The concept of an earthworm-day integrates survival rates, dilution factors, contaminant concentrations and time. As used in this thesis, one earthworm-day is defined as one worm surviving for one day. Earthworm-days were calculated as the cumulative survival days for each worm summed over all worms. For example, 30 worms all surviving a 14 day time period would correspond to 420 earthworm-days. Worm-days were calculated for all dilutions in all soils, and are presented in Figures 3.6 and 3.7.

3.4.3.1 Earthworm-days and TEO

Worms in the respective control soils for EDM and PAC achieved maximum survival. The worms exposed to low contaminant concentrations, in the PAL soil, survived for > 97 % of the maximum possible earthworm-days. Figure 3.6 illustrates a very sharp initial drop in worm-days followed by a slower progression toward zero

as the percent of TEO increased in EDM and PAC soils. There was only a slight separation between the EDM and PAC soils for the range of TEO used here. The differences in soils were greater at lower concentrations of TEO.

When TEO concentrations were less than 0.1 %, the current Alberta criterion, earthworm survival declined to less than 30 % for EDM (3.13 %) soil [TEO = 0.050 ± 0.003 %] and less than 13% for PAC (3.13 %) soil [TEO = 0.029 ± 0.002 %], of the maximum possible earthworm-days. One exception to this trend was the PAL soil. The PAL (100 %) soil had a TEO concentration of 0.043 ± 0.004 %, similar to both the EDM (3.13 %) and PAC (3.13 %) soils but worms still achieved maximum possible survival. Variable toxicity responses with sub-criterion TEO concentrations suggest there could be something in the EDM or PAC soil that is not in the PAL. Potentially, an acutely toxic compound or mixture of compounds in the EDM and PAC soils was the cause for the observed acute toxicity. Since the EDM and PAC sites operated in different locations at different times and creosote is such a variable mixture, the potential compound(s) imparting acute toxicity could be different for each soil.

The EDM biotreated soil was a major exception to the trend of increasing toxicity with increasing contamination. After 52 days of biotreatment acute toxicity appeared to be removed, even though the TEO content would predict earthworm survival to reach a lower percent of the earthworm-day maximum. The EDM (50%) soil had a TEO concentration of 0.76 ± 0.01 % but achieved only 7.3% of maximum earthworm-days. After 51 days of exposure to the EDM-Bio soil [TEO = 0.87 ± 0.01 %] the worms were still at 100 % of their maximum potential of earthworm-days, for that time period. The potential for the worms to survive for 52 days in the EDM soil after biotreatment suggests that the biotreatment was able to reduce the concentration of compounds, which may have produced the observed acute toxicity in the untreated EDM soils, to concentrations where there was no observed acute toxicity.
Total extractable organic contents in PAC-Bio and PAC (100 %) were not significantly different from one another and neither were the earthworm responses. Biotreatment of PAC was unable to remove the acute toxicity of the original PAC 100

% soil, in contrast to the EDM and EDM-Bio soil results. A potential explanation for the inability of microbes, in the PAC biotreatment, to reduce contaminant concentrations and remove acute toxicity may lie in the nature of the soil. The EDM soil has a clay texture, which has increased surface area, and may provide additional surfaces for microbial attachment relative to the PAC soil. Further, these increased surfaces could be potential sites for contaminates to sorb onto, thereby attenuating the contaminant and reducing bioavilability. The PAC soil has a sandy texture, which would have reduced surface area compared to the clay soil and therefore may not provide a suitable environment for contaminant degrading microbes to live, ultimately resulting in continued acute toxicity. Further, the EDM-Bio soil did have a significant decrease in the TEO concentration compared to EDM 100 % while the PAC-Bio did not reveal a significant decrease in TEO concentration. Alternatively, there could have been compounds in the PAC soil that were acutely toxic to the microbial population and essentially killed any hydrocarbon degrading microorganisms.

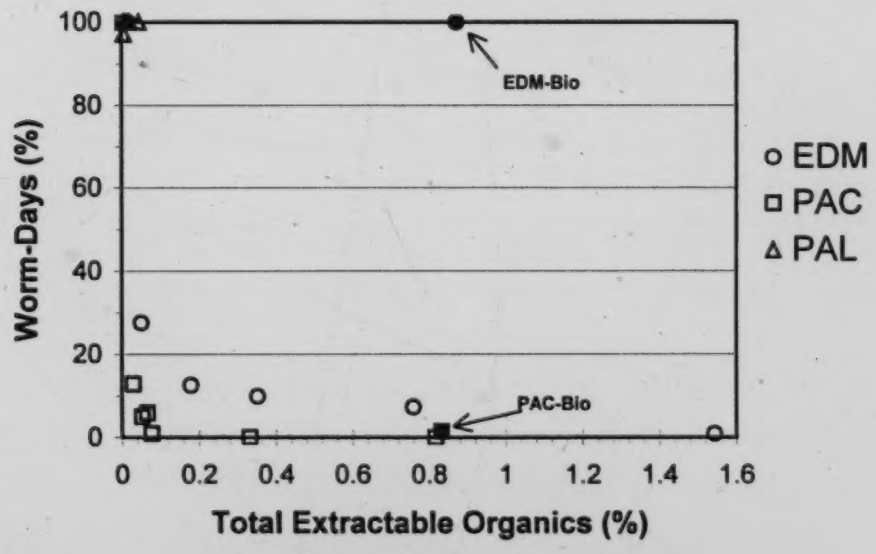

Figure 3.6: Relation of percent of maximum earthworm days during 14 day acute earthworm bioassay conditions with the total extractable organic content. Maximum potential worm-days were 420 for each soil treatment, except the biotreated soil, which was 140 worm-days.

3.4.3.2 Earthworm-days and Total PAHs

Total extractable organic values yield data on the total quantities of extractable organic material but they do not provide the identities of compounds within a mixture. To identify the compounds within creosote extracts, a GC/MSD quantification of the largest group of compounds found within creosote was conducted. As a class, PAHs represent 85 % (w/w) of the components which can make up a creosote mixture, excluding the carrier (Mueller et al., 1989). It was hypothesized that some PAHs may have correlated with the constituents that contributed to the observed acute toxicity found in the EDM, PAC, and PAC-Bio soils. The potential for PAHs to act as carcinogenic, mutagenic, and genotoxic agents also prompted further investigation into the relationship between 16 individual PAHs and earthworm toxicity. Percent earthworm-days was plotted against the total PAH concentration (Figure 3.7). Total PAHs was represented by the sum of 16 individual priority PAHs (Section 3.3.4). As observed in the TEO data (Section 3.4.3.1), an increasing quantity of PAHs resulted in a reduction of the percentage of maximum earthworm-days achieved by the worms in the toxicity tests. There was only a slight separation between the EDM and PAC soils for the range of total PAHs analyzed.

The EDM-Bio soil had a significant decrease in total PAH concentration, relative to the EDM soil, and maximum earthworm survival was achieved. The EDM 3.13 % soil had a lower total PAH concentration than EDM-Bio but reached a lower percentage of maximum earthworm-days. Again, this suggests that the biotreatment process was responsible for removing acutely toxic compounds, including PAHs of varying atomic mass. The PAC-Bio soil did not have a significant reduction of total PAHs and the percentage of maximum earthworm-days was not different from untreated PAC soil with a comparable level of total PAHs.

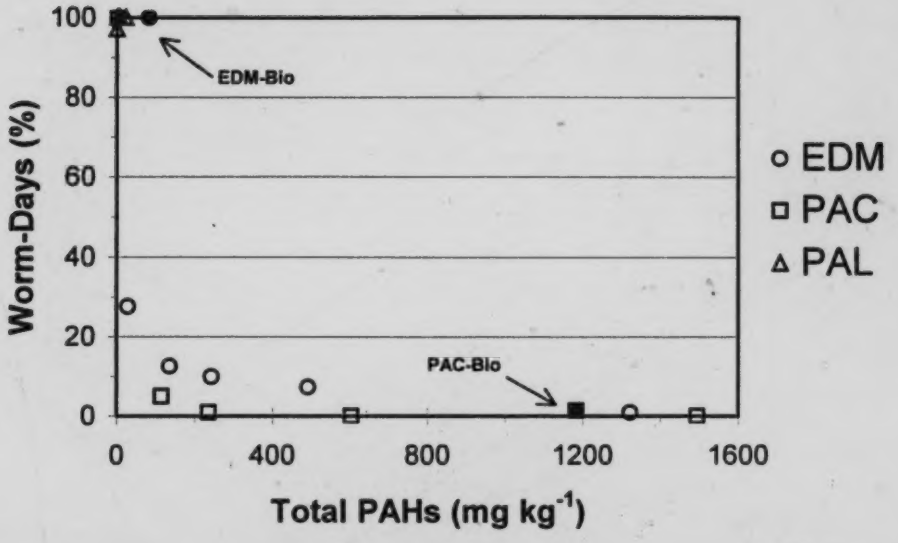

Figure 3.7: Relation of percent of maximum earthworm days during 14 day acute earthworm bioassay conditions with the total PAH content. Maximum potential worm-days were 420 for each soil treatment except the biotreated soil, which was 140 worm-days. Total PAH content was the sum of 16 quantified PAHs.

3.4.3.3. PAH / TEO Relations

Partitioning of contaminants into soil organic matter (SOM) is often thought of as a mediating parameter of toxicity in soils (Weissenfels et al., 1992). More recent experimental results suggest that the presence of a nonaqueous phase liquid (NAPL) may be even more important in controlling sorption and desorption mechanisms of PAHs than SOM (Rutherford et al., 1998). The NAPL present in soil could be functioning as a sink for toxic contaminants, and thereby decreasing the bioavailability of toxicants. As persistent organic chemicals age in the soil their potential to be biodegraded as well as their bioavailability to microbes (Hatzinger and Alexander, 1995) and to earthworms (Kelsey et al., 1997; Kelsey and Alexander, 1997) can decline.

Roos et al. (1996) examined the bioavailability of PAHs in contaminated soils to rats as well as the bacterium *Vibrio fischeri*. However, total PAHs did not correlate well with the measured toxicity to *V. fischeri*. Using a PAH / TOC (total organic carbon) ratio Roos et al. (1996) observed that toxicity, as measured by

bioluminescence inhibition, increased linearly as the PAH / TOC ratio increased. This trend suggests TOC may retard the release of the PAHs, and thereby decrease bioavailability and the measured toxicity in bacteria. Caution must be exercised when making cross species extrapolations, as mammalian bioavailabilty of soil bound PAHs may be only marginally influenced by soil properties (Roos et al., 1996). Ratios of total PAHs / TEO (Table 3.2) were used to evaluate the toxicity of weathered creosote contaminated soils to *E. fetida*. The EDM-Bio and PAL soils had the lowest PAH / TEO ratios as well as the lowest observed mortality, when compared to PAC-100% and PAC-Bio soil treatments. The EDM-100 % soil had an intermediate PAH / TEO ratio. Worms exposed to EDM-100 % reached a higher number of earthworm-days compared to PAC.

3.4.4 Michaelis-Menten Equation as a Model to Predict Earthworm-days

Equations depicting relationships between worm-days and either TEO or total PAHs were used to describe the EDM, PAC or Pooled (EDM and PAC together) soils (Table 3.3). The worm-day (WD) value is derived from the survival of earthworms during a toxicity test, in contrast to worm mortality (WM) which is the mirror image of worm-days. Worm mortality is derived (Equation 3.6) when the number of worm-days survived in a treatment is subtracted from the maximum number of possible earthworm-days (420). The value 420 is the maximum possible survival, in earthworm-days, of 30 worms surviving the entire 14 days of an acute toxicity test. The general form of the Michaelis-Menton equation for predicting worm mortality (WM) is given in Equation 3.7, where WMmax is the maximum predicted worm mortality, K_C is the concentration of contaminant that kills 50 % of the earthworms and C is either the concentration of TEO or total PAHs. The K_C parameter is therefore called the LC_{50}. Two methods for evaluating LC_{50} can be used, either a time endpoint or a survival time approach (Newman and Dixon, 1996). The predominant approach is the time endpoint, which describes toxicity in terms of a dose-response over a predetermined time period, for example 14 days. However, the survival time approach incorporates many more time data points over the course of the toxicity test. The survival time approach was used here by measuring earthworm days and

reporting toxicity. Parameter values for Equation 3.7 were calculated from both the TEO and PAH data using a nonlinear least squares analysis on the SAS System (1996) and reported in Table 3.3.

$$WM = 420 - WD \qquad \textbf{Where: WD = Earthworm-days} \qquad \text{(Equation 3.6)}$$

$$WM = \frac{WM_{max} \times C}{(K_C + C)} \qquad \textbf{Where: C = TEO (\%) or PAH (mg kg}^{-1}\textbf{)} \qquad \text{(Equation 3.7)}$$

Table 3.3: Michaelis-Menten parameters for predicting worm mortality in EDM and PAC soils. WM_{max} and K_c values were determined using a NLIN procedure in SAS. Predicted parameters (n=3) are given with upper and lower 95% confidence intervals.

Treatment	Estimated Parameters WM_{max} (worm mortality days)	K_c (Calc LC_{50})	R^2	95% Confidence Intervals Interval Overlap
EDM-TEO	436 ±50	0.042 ± 0.022 %	0.973	Yes
PAC-TEO	468 ± 53	0.015 ± 0.009 %	0.976	Yes
EDM-PAH	401 ± 7.1	10.4 ± 1.7 mg kg^{-1}	0.999	No
PAC-PAH	425 ± 1.8	6.92 ± 0.46 mg kg	0.999	No
Pooled-TEO	87.2 ± 52	-0.04 ± 0.005 %	0.388	ND
Pooled-PAH	415 ± 8.5	9.14 ± 2.1 mg kg^{-1}	0.998	ND

ND = Not determined

Correlation values were high ($R^2 \geq 0.97$) for the individual soils using either TEO or total PAH concentrations. The Michaelis-Menton equation was able to predict the worm-days using the pooled PAH data ($R^2 = 0.998$). The pooled data for TEO, yielded an equation with low predictive ability ($R^2 = 0.39$). Graphically, the results of Equation 3.7 are shown in Figure 3.8. The overlapping of the 95 % confidence intervals between the EDM and PAC soils for both WMmax and K_c indicating no differences between soils when using TEO to predict WMmax or K_c. In contrast, when using total PAHs to predict WMmax and K_c, the 95 % confidence intervals did not overlap between EDM and PAC for either parameter, indicating the soils where different.

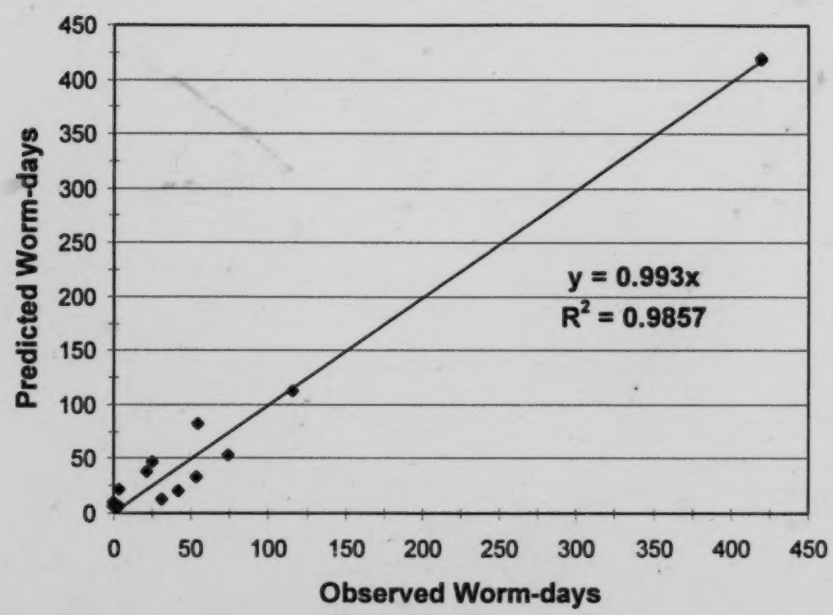

Figure 3.8: Testing of equation 3.7 using PAH concentrations; observed versus predicted worm-days for EDM and PAC soils. Missing PAH concentrations were predicted using equations developed in Chapter 2 for PAH concentrations and dilution factors.

3.5 Conclusions

Biotreatment was able to reduce the observed acute toxicity in EDM-Bio but not in PAC-Bio. Therefore, soils that have gone through a biotreatment process may remain acutely toxic to *E. fetidia*. Concentrations of total PAHs were reduced to a greater extent compared to the TEO concentration for the EDM-Bio soil. Unfavorable microbial habitat could be one reason the sandy soil did not experience the same reduction in PAHs or TEO, as the clay soil did.

It is clear that the sole use of TEO or total PAH concentration data can not precisely predict the eventual toxic consequences of creosote contamination to soil organisms such as *E. fetidia*. For example, the EDM-Bio and PAL soils were contaminated above Alberta Tier I Criteria but were not acutely toxic to *E. fetida*. Toxicity predictions based solely on analytical chemical data may poorly correlate to

measured toxicity based on biological determinations, as was also evident with the EDM-Bio soil.

The PAH data were more closely related to toxicity than the TEO, for the soils used here. However, TEO seems to have an essential role in the regulation of toxicity, as expressed in the PAH / TEO ratio. Soils with low PAH / TEO ratios (EDM-Bio and PAL) were less toxic, as measured by earthworm days compared to soils with higher ratios (EDM, PAC, and PAC-Bio). Bioavailability of organic toxicants, such as PAHs, appears to be reduced in the presence of a residual NAPL, at least for the soils studied here. There is potential for using the PAH / TEO ratio for *E. fetida* as a better predictor of toxicity compared to total PAH or TEO concentrations alone, however specific experiments need to be conducted to test this conjecture.

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Chapter 4: Subchronic Ecotoxicity Testing

4.1 Introduction

Some effort has been made to standardize acute soil toxicity bioassays. Efforts must also be made to evaluate potentially toxic outcomes after chronic exposures. However, chronic assays with mutagenic endpoints, for soil biota, have not historically received much research attention (Sheppard et al., 1992). Crude estimates of toxicity come from studies designed to evaluate a compound or mixture's LC_{50}. If an acute assay suggests a substrate is not acutely toxic, there could still be potential for chronic sublethal outcomes, which need to be evaluated. Techniques in genetic ecotoxicology are beginning to develop rapidly (Anderson et al., 1994). Although genetic ecotoxicology is more than just the study of direct DNA damage from exposure to environmental toxicants (Anderson et al., 1994), experiments in this chapter focused entirely on DNA adduct formation.

One approach to environmental monitoring is the use of biological markers. The use of biomarkers provides a potentially sensitive technique when assessing exposure, effect, or susceptibility of organisms to contaminants. Biomarkers "...reflect molecular and/or cellular alterations that occur along the temporal and mechanistic pathways connecting ambient exposure to a toxicant and eventual disease" (Decaprio, 1997, p.1837). Use of biomarker data occurs in many disciplines such as occupational health, risk assessment, epidemiology and ecotoxicology. Protein and DNA adducts as well as metallothioneins have been used as biomarkers of exposure to measure the internal doses of xenobiotics. Biomarkers of exposure can measure the internal dose of a chemical, thus providing information on bioavailability (Decaprio, 1997). Biomarkers of effect are predictive precursors of toxicity and health impairments resulting from exposure to potentially damaging agents (Decaprio, 1997). Examples of biomarkers of effect include oncogene activation, sister chromatid exchange, and single strand DNA breakage. Susceptibility biomarkers measure the rate of transition between steps along the exposure / disease continuum.

Activities of enzyme systems such as glutathione-S-transferase and cytochrome P-450 provide a measure of biological susceptibility (Decaprio, 1997).

Current government guidelines address total contaminant concentrations. However, this external dose only represents the solvent-extractable portion of a chemical, or mixture of chemicals. Little consideration has been given to biologically relevant doses. A biologically available dose can range from 100% of the chemically extractable fraction to only a very small percentage of the solvent-extractable compound (Kendall et al., 1996). Once an organic contaminant is introduced into a soil environment, there are sorption/desorption mechanisms regulating its environmental fate in the soil, ground water, and air. Soil physical-chemical properties such as pH, organic matter content and clay content can also modify the bioavailability of a compound (van Schooten et al., 1995). Furthermore, if contaminated soil contacts an organism, either through inhalation, ingestion, or dermal exposure; several physiological barriers must be crossed before a contaminant can become biologically available. Thus a measurement of bioavailability is useful because it integrates many mechanisms and processes along a continuum which ultimately results in a chemical reaching a target site.

Compounds such as PAHs, which are found in creosote, can be bioactivated *in vivo* by naturally occurring enzymes to reactive metabolites that bind to macromolecules such as DNA. Many carcinogens are electrophilic and can covalently react with DNA (Legator and Au, 1994). Benzo[a]pyrene (BaP) is a classic example of a PAH that is metabolized by enzymes into reactive epoxide metabolites. Epoxidation reactions are catalyzed by monooxygenases such as cytochrome P-450 enzymes, while the conversion from an epoxide to a dihydrodiol species is mediated by epoxide hydrolase (Figure 4.1). Benzo[a]pyrene 7,8-dihydrodiol-9,10-epoxide is one of the ultimate metabolites of BaP that binds to DNA (Cerniglia, 1984; Stowers and Anderson, 1985; Parkinson, 1996). In mammals and fish, cytochrome P-450 has been identified as an activator of procarcinogenic PAHs (van Schooten et al., 1995). In worms, some information suggests cytochrome P-450 can be extracted from microsomes in midgut of *Lumbricus terrestris*. However, it has

not been clearly established if worms bioactivate PAHs into electrophilic forms (van Schooten et al., 1995).

Reactive chemical species such as benzo[a]pyrene 7,8-dihydrodiol-9,10-epoxide, can form addition products or adducts, which if left unrepaired can result in genetic mutations during DNA replication (Gupta, 1993). Various types of DNA damage have been quantified by several techniques such as ^{32}P postlabeling (Walsh et al., 1995a; Walsh et al., 1997), HPLC and GC / MS (Cadet and Weinfeld, 1993), as well as an ultrasensitive assay utilizing capillary electrophoresis coupled with laser-induced fluorescence detection (Le et al., 1998). DNA adduct formation is considered a critical step in the initiation of carcinogenesis of humans (Gupta, 1985). DNA adducts have also been measured in several organisms that have been exposed to PAHs such as: white sucker fish (*Catostomus commersoni*) (El Adlouni et al., 1995b); brown bullheads (*Ictalurus nebulosus*) (Dunn et al., 1987); eels (*Anguilla anguilla*) (van Schooten et al., 1995); and earthworms (*Lumbricus terrestris*) (van Schooten et al., 1995; Walsh et al., 1995a; Walsh et al., 1995b), (*Eisenia fetida*) (Walsh et al., 1997). Quantification of DNA adducts can provide a measure of exposure to environmentally sensitive organisms. Cross species interpretation of DNA adduct results must be done carefully.

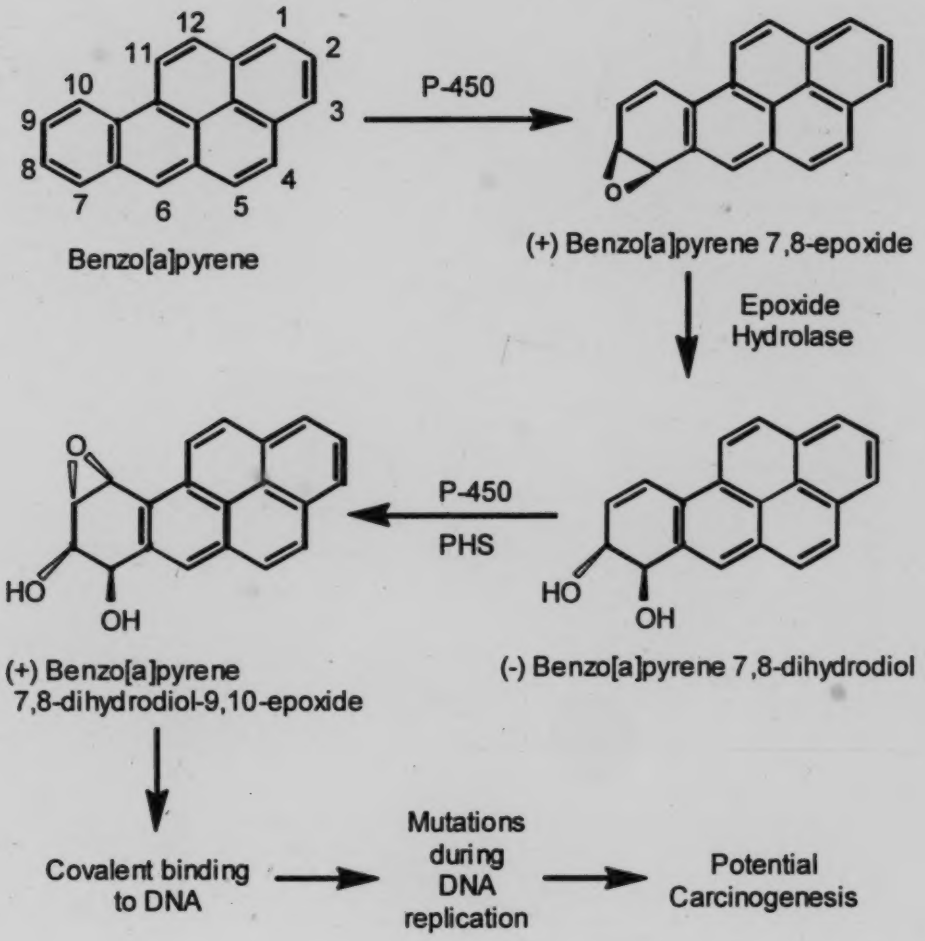


Figure 4.1: Formation of the reactive (+) benzo[a]pyrene 7,8-dihydrodiol-9,10-epoxide. PHS = prostaglandin H synthase (modified from Parkinson, 1996).

4.2 Objective

The objectives of the work reported in this chapter were threefold. Firstly, to determine DNA adduct quantities in earthworms exposed to weathered creosote contaminated soils without BaP additions or biotreatment. Secondly, determine if the biotreatment of the EDM soil would reduce subchronic toxicity, as measured by the formation of DNA adducts in *E. fetida*. Thirdly, to determine whether earthworm exposure to elevated concentrations of PAHs, specifically BaP, would result in increased DNA adduct formation in *E. fetida*. Subchronic exposure periods for the earthworms ranged from 27 to 49 days.

4.3 Materials and Methods

4.3.1 Experimental Protocol

Use of the term contaminated soil, in this thesis, refers to an admixture of contaminated soil horizons and PGM. Contaminated soils for subchronic toxicity testing were selected based on earthworm survival data described in Chapter 3 (Acute Ecotoxicity Testing). A soil was included in subchronic ecotoxicity testing if earthworm survival was 100 % over 14 consecutive days. One biotreated soil (EDM-Bio) and one weathered creosote contaminated soil (PAL) were selected based on contaminant content and toxicity properties, as potential soils that could impart a subchronic toxicity response, in terms of increased DNA adduct formation in *Eisenia fetida*. Two pristine control soils (Malmo-C and Brud-C) were also included as controls in the subchronic toxicity tests.

There were eight different soil treatments in total. Four treatments used the PAL soil in combination with additional quantities of BaP (high, medium, low, and zero concentrations) that were applied in dichloromethane (DCM) as a carrier solvent. The fifth treatment used only PAL soil, without additional BaP or solvent. The sixth treatment used Brud-C and only DCM. In treatment seven, worms were exposed to the EDM-Bio soil. No solvent was added to the EDM-Bio soils. Treatment eight, a negative control for the EDM-Bio soil, was the Malmo-C soil without BaP or DCM.

All treatments were completed in triplicate except treatments seven and eight. Treatment seven was conducted in duplicate because of limited quantities of soil available. Treatment eight was conducted in duplicate because there was strong background evidence on the behavior of the Malmo-C soil, from the acute toxicity tests; additionally treatment seven could only be completed in duplicate, so its control was also duplicated.

4.3.1.1 Earthworms

Eisenia fetida from the Carolina Biological Supply Company (Burlington, NC) were used in all subchronic toxicity tests. The earthworms were mature, with clitellum. The overall mean earthworm mass for the subchronic testing was 375.7 ± 61.7 mg. Earthworm masses ranged from 300.0 to 628.4 mg. As described previously (Section 3.3.3), the earthworms were maintained under moist conditions within a growth chamber in 15.5 L translucent, covered, plaster containers (Rubbermaid) under constant light (4004 ± 193 lux) and temperature (22 ± 2°C).

4.3.1.2 Benzo[a]pyrene Additions

Soils used in the subchronic ecotoxicity tests are characterized in detail in Chapter 2. The soils used for the subchronic testing were: Brud-C, Malmo-C, PAL, and EDM-Bio. The PAL soil, which had lower concentrations of TEO and PAHs, compared to the PAC and EDM soils, was selected for a BaP spiking experiment because earthworms were able to survive extended exposures to PAL and because some residual non-aqueous phase liquid (NAPL) would be present in the soil. A soil with residual NAPL, was thought to be better for receiving a hydrophobic contaminant spike compared to a soil without a NAPL phase because the NAPL phase would act as a partitioning phase for the additional BaP, thus modeling how a weathered creosote contaminated soil might behave. Non-aqueous phase liquids have been shown to modify toxicity responses in microbes during bioremediation processes (Rutherford et al., 1998).

Benzo[a]pyrene (Min 97 % HPLC, Sigma-Aldrich; Lot 107H3569) was dissolved in dichloromethane (Fisher Scientific Co. Optima Grade) which produced a

stock solution of 6000 mg L^{-1}. The stock solution was serially diluted to yield concentrations of 6000 mg L^{-1}, 600 mg L^{-1}, and 60 mg L^{-1}. These BaP solutions (50 ml) were applied to 300 g (oven dry weight) samples of PAL, thereby increasing soil BaP concentrations an additional 1000 mg kg^{-1}, 100 mg kg^{-1}, and 10 mg kg^{-1}. There were also three controls: PAL with only DCM; PAL with no solvent or BaP; and Brud-C, a pristine control soil, which only had DCM added. The EDM-Bio and Malmo soils were included in the subchronic toxicity testing but they had neither DCM nor additional BaP additions. Moreover, the soils were not homogenized for 14 days using the roller bottle apparatus, as treatments 1 - 6 were.

The BaP solutions (50 ml) were added to soils in 1 L earthworm incubation vessels, which consisted of 1 L wide mouth mason jars (Bernardin) with snap lids. The lids for all jars had two ¼ inch (6.35 mm) holes drilled into the top, allowing the DCM to escape. The snap lids each had a piece of filter paper (Whatman Qualitative No. 2, 9 cm diameter) placed underneath to prevent soil from escaping through the holes in the lids, while being homogenized on a roller bottle apparatus (3.7 rpm). After the BaP and DCM additions, the vessels were kept in a fumehood (22 ± 1°C) for 24 hours without lids, which allowed the majority of the solvent carrier to evaporate. The soils were then homogenized and any remaining DCM vented by placing each vessel horizontally on a rollerbottle apparatus where they were continuously rotated (3.7 rpm) for 14 days.

Table 4.1: Summary and comparison of contaminant characteristics from the core creosote contaminated and biotreated and soils to Alberta Tier I guidelines.

Soil	Total PAHs (mg kg^{-1})	TEO (%)	PAH / TEO	Analyze worms for Adducts?
PAL-HI	1020*	0.043 ± 0.004	2.4	Yes
PAL-MED	120*	0.043 ± 0.004	0.28	No
PAL-LOW	30*	0.043 ± 0.004	0.070	Yes
PAL	20 ± 5†	0.043 ± 0.004	0.047	Yes
EDM-Bio^,‡	80 ± 10†	0.87 ± 0.01	0.0092	Yes
Brud-DCM	0 ± 0†	0.005 ± 0.003	0.00	Yes
Malmo-C	0 ± 0†	0.016 ± 0.002	0.00	No
Alberta Tier I	1.0	0.1	0.001"	NA

*Note total PAHs is defined as the sum of 16 PAHs as quantified by GC/MSD plus 1000, 100, or 10 mg kg^{-1} (High, Med, and Low respectively) of BaP.

†Total PAHs is defined as the sum of 16 PAHs as quantified by GC/MSD.

^Denotes soil after 52 days of biotreatment.

‡Earthworms exposed for 49 days were started in the acute toxicity testing (Chapter 3) and remained in the assay vessel until day 49.

"This parameter is not actually part of the Alberta Tier I guidelines.

NA = Not applicable.

4.3.1.3 Subchronic Toxicity Testing

Preparation of homogenized soils (PAL, EDM-Bio, Malmo-C and Brud-C) for the subchronic toxicity tests followed the procedure for soil preparation as outlined in Section 3.3.5.2. A portion of each homogenized soil (30 – 50 g) was removed from the earthworm incubation vessels and a gravimeteric moisture content was determined (Equation 3.1). As before (Section 3.3.5.2) the required mass of water (DDW) required to bring the soils to 60 % water filled pore space (WFP) was calculated. Water was applied as a fine mist to the homogenized soil surface in each vessel, using a spray bottle. The soils were allowed to sit overnight, and the following day the soils were stirred with a metal spatula and then individually weighed earthworms were added onto the homogenized soil surface. The incubation vessels were arranged randomly and stored in a fumehood under constant temperature (22 ± 1°C) and lighted conditions (810 ± 120 lux). No effort was made to force the earthworms to burrow into the soil. A fine mist of water was added to the surface of the soils, as required, thereby maintaining a water content of 60 % WFP, throughout the experiment. The worms were monitored initially for 14 days, at which time half of all

live worms from each vessel were removed and subsequently stored in liquid nitrogen. The experiment proceeded as before with periodical monitoring of worms until day 27.

When live earthworms were removed from the test soils, they were placed in sterilized, plastic Petri dishes (100 X 15 mm) (Fisher Scientific Co.). Each Petri dish contained two pieces of filter paper (Whatman Qualitative No. 1, 9 cm) that were moistened with DDW. The earthworms were sandwiched between the two pieces of moist filter paper and the dishes were taped shut, to prevent earthworms from escaping. The worms in the dishes were stored in the dark at 22°C for 48 hours, which allowed time for the worms to evacuate the contents of their intestinal tracts. The evacuation procedure was conducted to reduce the quantities of soil and foreign DNA within the earthworm intestine, which could be a confounding factor in the quantification of DNA adducts (Dr. D. Nadeau, personal communication). Earthworms exposed to the EDM-Bio soil treatment for 49 days (Chapter 3) were evacuated at an earlier date, before the BaP spiking experiment. The evacuation procedure was the same as described above. The EDM-Bio (49 day) worms were individually frozen, placed in plastic vials (7 ml) and then stored submerged in liquid nitrogen for approximately 2 months, to prevent tissue breakdown.

After the BaP spiking experiment and all the worms had been evacuated, they were frozen in liquid nitrogen. All worms were stored individually in plastic vials (7 ml), which were kept submerged in liquid nitrogen until the samples were shipped to the University of Laval (Sainte-Foy, Québec) for DNA adduct quantification. Earthworm samples were packed in dry ice and shipped via air courier for overnight delivery.

4.3.2 Preparation of DNA

Ms. Rashmi Shah (Unité de recherche en santé et environnement (Health and Environment Unit), Centre de recherche du Centre Hospitalier de l'Université Laval (CHUL); Sainte-Foy, Québec) performed all DNA extractions and DNA quantifications under the direction of Drs. D. Nadeau and G.G. Poirier.

All chemicals were reagent grade, obtained from either Sigma-Aldrich or Fisher Scientific Company unless otherwise indicated. Phenol used was obtained from Gibco-BRL and Chloroform (HPLC grade) was from Fisher Scientific Company. Pipet tips and microcentrifuge tubes (polypropylene) were autoclaved for 15 minutes at 121°C. Solutions were made with HPLC grade water (treated with UV light against pyrogens) at 18.2 Megaohms.

4.3.2.1 DNA Extraction and Purification

Genomic DNA was isolated from *E. fetida* as described by El Adlouni et al. (1995a). The DNA extraction procedure with modifications is summarized in Figure 4.2. A detailed, but not exhaustive, record of the method can be found in Appendix B. For a comprehensive source of molecular techniques and general methodology information see Sambrook et al. (1989). The principles of the method are set out here.

One of the most fundamental molecular techniques is the purification of nucleic acids (Sambrook et al., 1989). After the earthworm tissue had been frozen with liquid nitrogen and homogenized physically, a series of enzymes and solvents were used to isolate the desired DNA from the unwanted proteins and RNA.

The enzymes and solvents used in the DNA extraction procedures (El Adlouni et al., 1995a) are typical of those used in many extraction protocols. Removal of proteins is a critical step in the purification process. Initially, earthworm tissue was incubated with 20 % sodium dodecyl sulfate (SDS), SET buffer (NaCl 100 mM; EDTA 20mM, Tris base 50 mM; pH 8.0) and proteinase K, which allows for the digestion, and ultimate removal of proteins from the tissue sample. Sodium dodecyl sulfate is a surfactant that can form micelles around polypeptide chains, thereby solubilizing the protein (Mathews and van Holde, 1990). Proteinase K is a proteolytic enzyme that is active against a broad spectrum of proteins, thereby aiding protein digestion in earthworm tissue.

The solvents used in the DNA extraction protocol (El Adlouni et al., 1995a; Walsh et al., 1995b) are important in removing unwanted material from the reaction mixtures as well as allowing the desired DNA to partition into a different solvent.

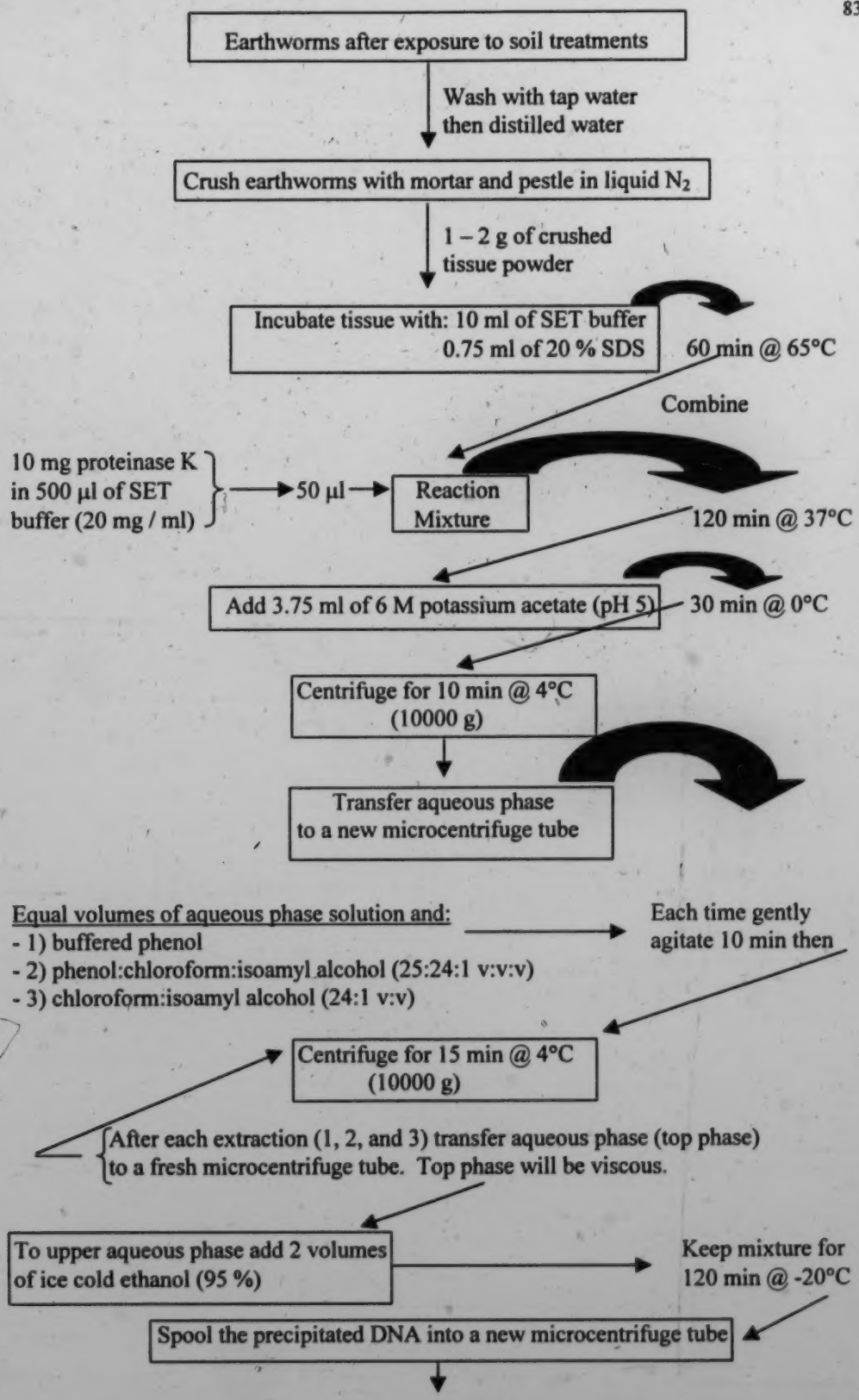
The solvents: phenol, phenol:chloroform:isoamyl alcohol (25:24:1 v:v:v) and chloroform:isoamyl alcohol (24:1 v:v) are important in extracting aqueous nucleic acids as well as inactivating enzymes (Sambrook et al., 1989). Proteins are removed from the reaction mixture with organic solvents. Phenol breaks hydrogen bonds, denatures proteins and is a solvent for RNA. Chloroform also denatures proteins and serves to separate the aqueous and organic phases (Sambrook et al., 1989). Further, chloroform removes any traces of phenol from the aqueous nucleic acid mixture. Isoamyl alcohol is used to reduce foaming during extractions. DNA and RNA are most commonly precipitated with ethanol. Use of ethanol is rapid and qualitative, even at picogram concentrations of DNA or RNA (Sambrook et al., 1989). Low temperatures (0°C) combined with controlled time and speed of centrifugation allow for the recovery of DNA.

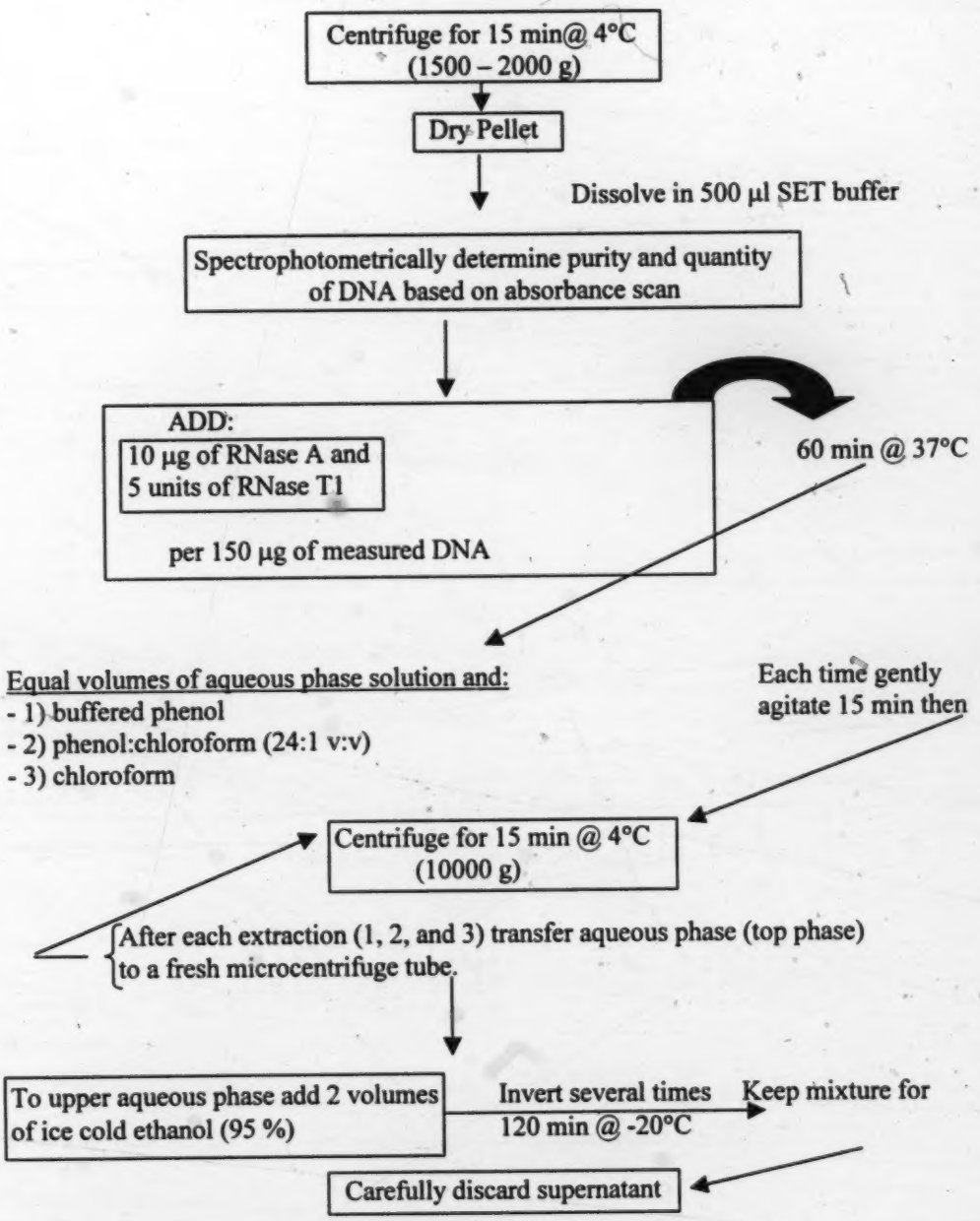
After proteins are removed from a tissue sample, the RNA is eliminated. An estimate of the concentration of DNA is used to determine the quantity of RNase enzymes to add. RNase A and RNase T1 are added to hydolyze RNA. Solvent partitioning again aided in further removing proteins and separating the aqueous nucleic acid from the organic protein / RNA phase.

4.3.2.2. DNA Quantity and Quality

The amount of DNA in solution can be determined spectrophotometrically (Sambrook et al., 1989). Absorbance measurements at 260 and 280 nm are required. Ultraviolet light, in the range of 270 - 290 nm is absorbed by aromatic amino acids, and at 260 nm is strongly absorbed by nucleic acids; therefore, proteins are commonly quantified by measuring absorption at 280 nm and DNA is quantified at 260 nm (Mathews and van Holde, 1990). At 260 nm an optical density (OD) of 1 corresponds to approximately 50 $\mu g\ ml^{-1}$ of double stranded DNA, considering the mass of a nucleotide pair in DNA is 660 daltons (Sambrook et al., 1989; El Adlouni et al., 1995a; Walsh et al., 1995a). The quantity of DNA measured determines the required quantity of RNase's necessary to fully react with the tissue reaction mixture (Section 4.3.2.1).

DNA quality is also an important consideration in the DNA extraction procedure. A ratio of OD_{260} / OD_{280} provides an estimate of nucleic acid purity. Pure DNA solutions will have an OD_{260} / OD_{280} value of 1.8, pure RNA has a value of 2.0 (Sambrook et al., 1989). If the OD_{260} / OD_{280} value is substantially less than 1.8, there is contamination with proteins or phenol. Further, a ratio greater than 1.8 suggests RNA contamination.


Earthworms after exposure to soil treatments
Wash with tap water then distilled water
Crush earthworms with mortar and pestle in liquid N_2
1 – 2 g of crushed tissue powder
Incubate tissue with: 10 ml of SET buffer 0.75 ml of 20 % SDS
60 min @ 65°C
Combine
10 mg proteinase K in 500 µl of SET buffer (20 mg / ml)
50 µl
Reaction Mixture
120 min @ 37°C
Add 3.75 ml of 6 M potassium acetate (pH 5)
30 min @ 0°C
Centrifuge for 10 min @ 4°C (10000 g)
Transfer aqueous phase to a new microcentrifuge tube
Equal volumes of aqueous phase solution and:
- 1) buffered phenol
- 2) phenol:chloroform:isoamyl alcohol (25:24:1 v:v:v)
- 3) chloroform:isoamyl alcohol (24:1 v:v)
Each time gently agitate 10 min then
Centrifuge for 15 min @ 4°C (10000 g)
After each extraction (1, 2, and 3) transfer aqueous phase (top phase) to a fresh microcentrifuge tube. Top phase will be viscous.
To upper aqueous phase add 2 volumes of ice cold ethanol (95 %)
Keep mixture for 120 min @ -20°C
Spool the precipitated DNA into a new microcentrifuge tube


Centrifuge for 15 min@ 4°C
(1500 – 2000 g)
Dry Pellet
Dissolve in 500 µl SET buffer
Spectrophotometrically determine purity and quantity of DNA based on absorbance scan
ADD:
10 µg of RNase A and
5 units of RNase T1
per 150 µg of measured DNA
60 min @ 37°C
Equal volumes of aqueous phase solution and:
- 1) buffered phenol
- 2) phenol:chloroform (24:1 v:v)
- 3) chloroform
Each time gently agitate 15 min then
Centrifuge for 15 min @ 4°C
(10000 g)
After each extraction (1, 2, and 3) transfer aqueous phase (top phase) to a fresh microcentrifuge tube.
To upper aqueous phase add 2 volumes of ice cold ethanol (95 %)
Invert several times
120 min @ -20°C
Keep mixture for
Carefully discard supernatant

Wash pellet of DNA 3X's (5 ml) with 70 % ethanol, asparate solution off
Dissolve pellet in SET buffer for storage

Figure 4.2: Summary of genomic DNA extraction procedure, with modifications, from *E. fetida* as described by El Adlouni et al. (1995).

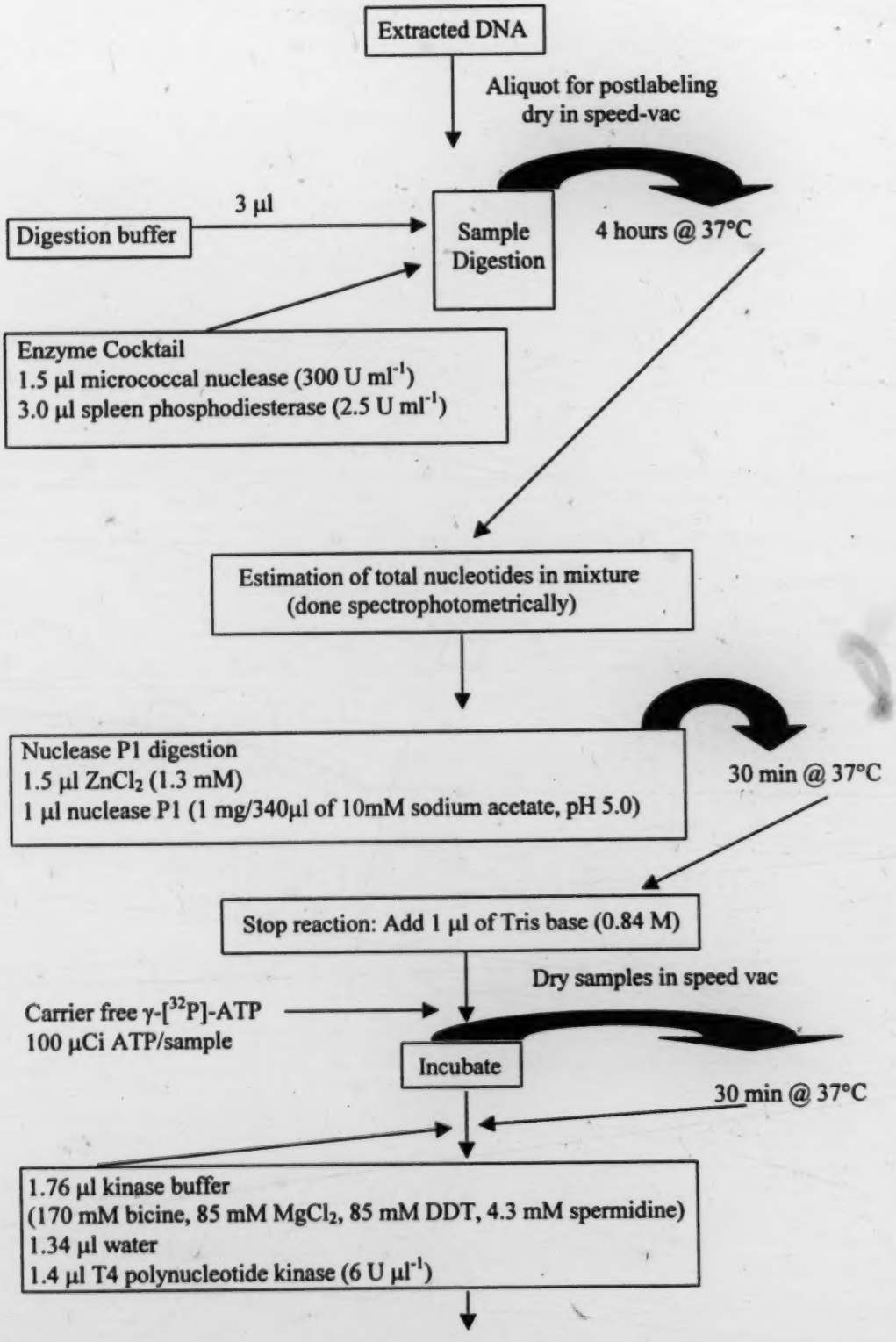
4.3.2.3 Postlabeling

A summary, with modifications, of the ^{32}P postlabeling protocol is provided in Figure 4.3. A detailed, but not exhaustive description of the the ^{32}P postlabeling procedure is provided in Appendix B of this thesis. Cadet and Weinfeld (1993) summarize the basic principles of the ^{32}P postlabeling assay that can be used to detect DNA adducts, which are interpreted as damage.

The postlabeling procedure measures DNA adducts after tissue exposure to a DNA modifying agent. The postlabeling technique allows nonradioactive chemicals to be used for exposure conditions, thus increasing the potential number of agents that can be tested, as well as limiting the amount of radioactive compounds used in the experiment (Randerath et al., 1981).

Potentially damaged DNA was extracted from earthworms (Section 4.3.2.1) and subsequently hydrolysed for postlabeling. An enzyme cocktail that includes micrococcal nuclease and spleen phosphodiesterase was used to hydrolyze the DNA (Figure 4.4). The cocktail of enzymes used hydrolyzes the DNA to nucleoside 3′-monophosphates, which generates free damaged bases with a high background of undamaged bases. To overcome the problem of background "noise", nuclease P1, is added to the reaction mixture. Nuclease P1 dephosphorylates the undamaged bases (nucleoside 3′- monophosphates i.e. undamaged deoxyribonucleotides) yielding deoxyribonucleosides, however, the damaged bases retain their phosphate group and remain as deoxynucleotides. The key to the technique is the enzyme used during the ^{32}P labeling, T4 polynucleotide kinase, which does not use nucleosides (purine or pyrimidine base plus a sugar (ribose)) as substrate, but rather nucleotides (purine or pyrimidine base, plus a sugar (ribose), and a phosphate ester). T4 polynucleotide kinase catalyzes the transfer of the γ-phosphate of ATP to a 5′ terminus of a deoxyribonucleotide (Sambrook et al., 1989). Only the damaged bases, with intact 3′ phosphate groups (nucleotides), are available to be radioactively phosphorylated in the 5′ position. The reaction mixture is then subjected to four dimensional (4-D) thin layer chromatography (TLC), which separates the DNA adducts from the excess

radiolabeled ATP and ADP. The radioactive spots on the TLC plates are then quantified by autoradiography and a quantity of DNA adducts per 10^9 nucleotides can be calculated.


Extracted DNA
Aliquot for postlabeling
dry in speed-vac
3 µl
Digestion buffer
Sample
Digestion
4 hours @ 37°C
Enzyme Cocktail
1.5 µl micrococcal nuclease (300 U ml-1)
3.0 µl spleen phosphodiesterase (2.5 U ml-1)
Estimation of total nucleotides in mixture
(done spectrophotometrically)
Nuclease P1 digestion
1.5 µl ZnCl2 (1.3 mM)
1 µl nuclease P1 (1 mg/340µl of 10mM sodium acetate, pH 5.0)
30 min @ 37°C
Stop reaction: Add 1 µl of Tris base (0.84 M)
Dry samples in speed vac
Carrier free γ-[32P]-ATP
100 µCi ATP/sample
Incubate
30 min @ 37°C
1.76 µl kinase buffer
(170 mM bicine, 85 mM MgCl2, 85 mM DDT, 4.3 mM spermidine)
1.34 µl water
1.4 µl T4 polynucleotide kinase (6 U µl-1)

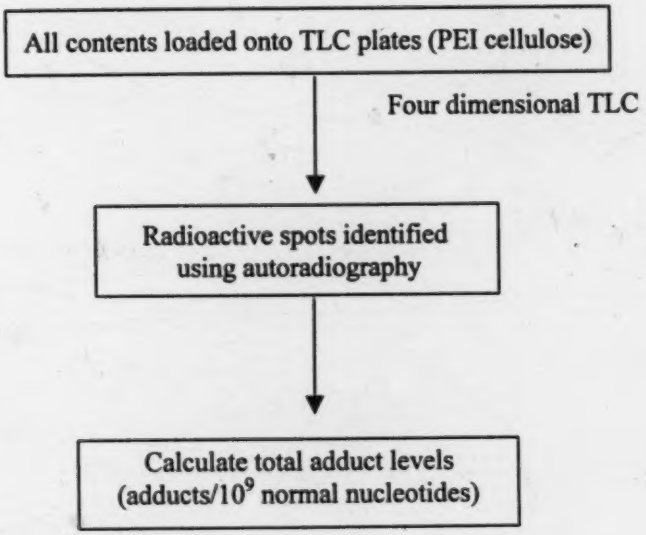

All contents loaded onto TLC plates (PEI cellulose)
Four dimensional TLC
Radioactive spots identified using autoradiography
Calculate total adduct levels (adducts/10^9 normal nucleotides)

Figure 4.3: Summary of ^{32}P postlabeling assay for bulky DNA adducts in *E. fetida*, with modifications, adapted from Reddy and Randerath (1986) and Walsh et al. (1995b).

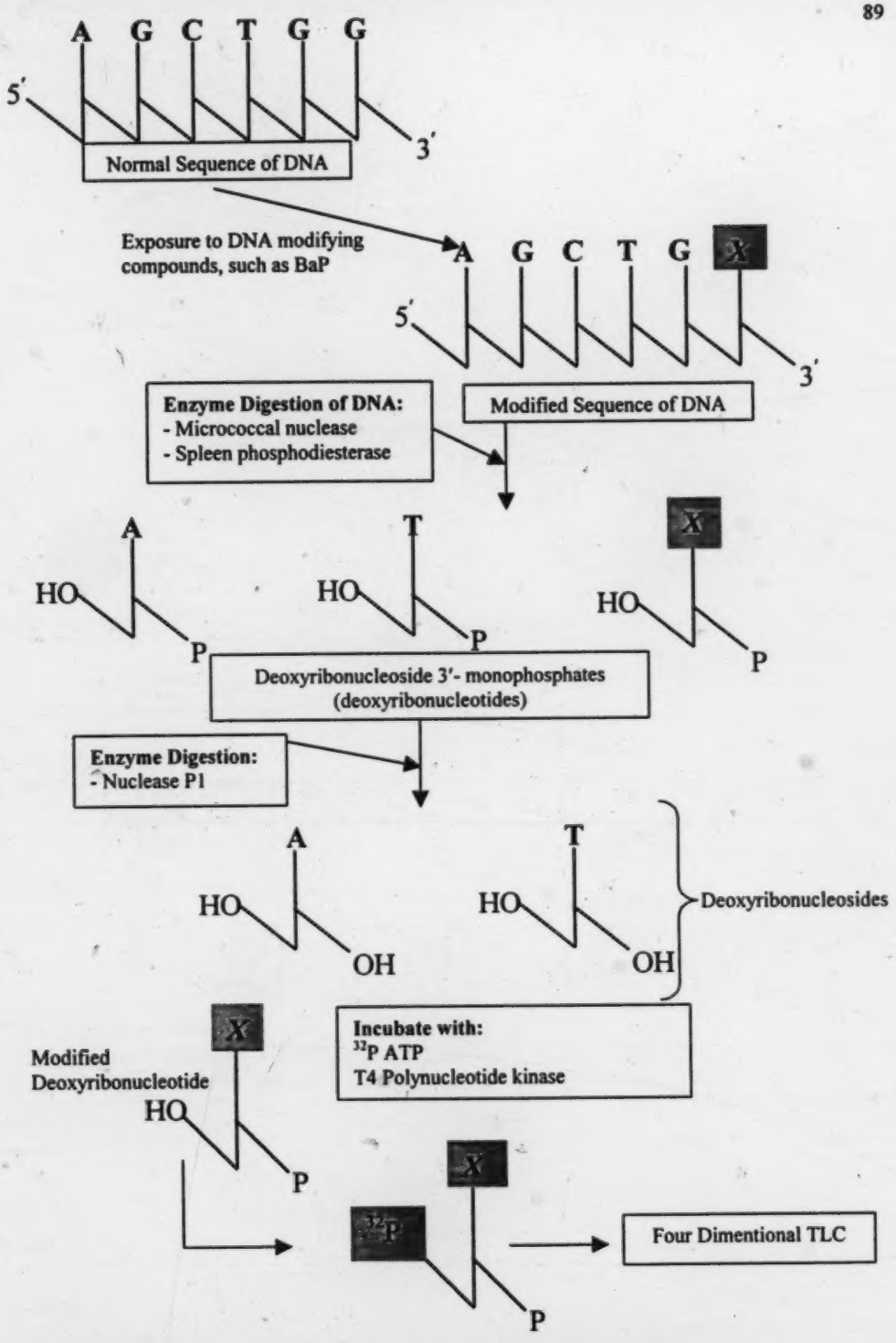


Figure 4.4: Basic principles involved in the ^{32}P Postlabeling procedure. A = Adenine; G = Guanine; C = Cytosine; T = Thymine; X = Modified base.

4.3.3 Statistical Analysis

Statistical analyses of earthworm adduct data were performed using the SAS System (SAS, 1996). Concentration of adducts from earthworms in seven soil treatments were compared using a general linear model (GLM) procedure. Significant differences between treatments were based on employing Duncan's multiple range test.

4.4 Results and Discussion

4.4.1 Earthworm Survival

Earthworm survival was recorded for the sub-chronic toxicity testing as the percentage of maximum earthworm days over varying PAH concentrations (Figure 4.5). All soils were chosen because the earthworms were able to survive at least 14 days in the acute toxicity tests (Chapter 3).

Over the first seven days all worms were burrowing into the soil and some worms were found in earthworm balls. On day five, two worms had died in the EDM-Bio soil. After day seven, some worms in the EDM-Bio soil were not burrowing into the soil. On day ten, two more worms had died. In all other soil treatments, all worms survived, and were buried for 14 days. On day 14, half of all live worms were removed from their respective soil treatments and stored in liquid nitrogen. After 27 days only 2 worms had survived in the EDM-Bio soil treatment.

No more than two worms had died in any PAL soil treatment, regardless of the quantity of BaP added, by day 27. No worm deaths occurred in the PAL treatments until at least day 18. In the PAL-Med treatment, all worms survived the entire test period. All worms survived in the two pristine soils, for 27 days.

Earthworm deaths in the subchronic test of the EDM-Bio soil were surprising because during the acute toxicity study, and for an additional 35 days after, all earthworms survived in the EDM-Bio soil. In contrast, during the subchronic testing, earthworm deaths began by the fifth day. Differences in the acute survival rate might be due to variability in worm stocks, although this is speculative. The PAL soil treatments did not show the same increase in observed acute toxicity as the EDM-Bio

soil did when compared to the earlier acute toxicity tests. In fact, earthworm survival in all PAL subchronic experiments closely paralleled the results, for the same time periods, in the PAL acute toxicity test, which continued for 41 days.

Table 4.2: Subchronic test soil characteristics.

Soil Designation	Solvent Added	Additional BaP (mg kg^{-1})	Exposure Times (days)
PAL-HI	DCM	1000	14 and 27
PAL-Med	DCM	100	27
PAL-Low	DCM	10	27
PAL-DCM	DCM	0	27
PAL-Z	None	0	27
Brud-DCM	DCM	0	27
EDM-Bio14	None	0	14
EDM-Bio49	None	0	49
Malmo-C	None	0	27

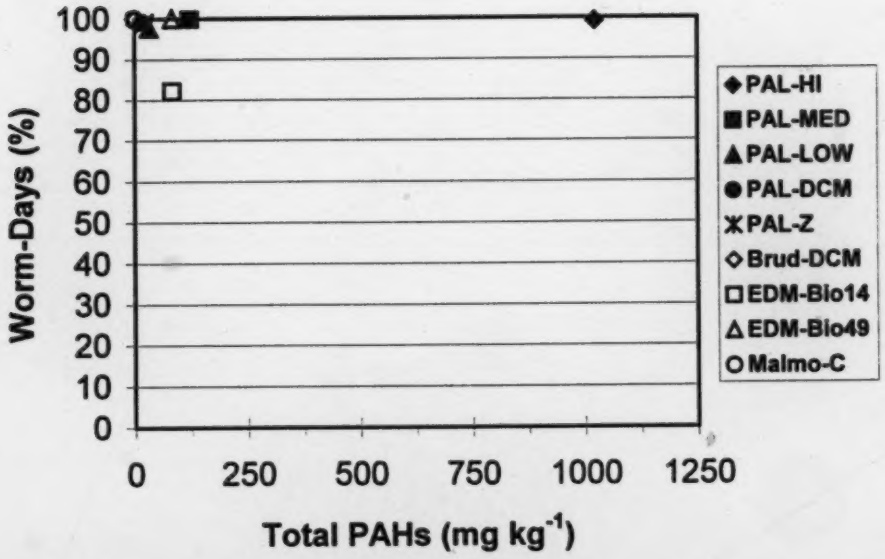


Figure 4.5: Relation of percent of maximum earthworm days during subchronic earthworm bioassay conditions with the total PAH concentration. Time of exposure for the subchronic experiments was 27 days in all treatments, except EDM-Bio49, when the exposure time was 49 days. Maximum potential worm-days were: 615 days for all PAL soils and Brud-DCM, 358 days for EDM-Bio14, 490 days for EDM-Bio49, and 410 days for Malmo C. Total PAH content is the sum of 16 quantified PAHs.

4.4.2 DNA Adducts

All adduct values are reported per 10^9 normal nucleotides. Two independent experiments (I and II) conducted on the same earthworm DNA extracts were used to quantify DNA adduct damage in *E. fetida* after exposure to weathered creosote soils, contaminated soils with additional quantities of BaP, and biotreated soils. Autoradiograms from the ^{32}P postlabeling procedure for six treatments are shown in Figure 4.6. The DNA adduct patterns were compared to the BaP control, an ATP control, and the pristine soil with no PAHs added. All controls were consistent with expected autoradiograms (Walsh et al., 1995b). The DNA adduct patterns in the contaminated soils showed a diffuse diagonal radioactive zone. The diagonal zone is consistent with adduct patterns after exposure to PAH mixtures (Gupta, 1993; El Adlouni et al., 1995; Walsh et al., 1997).

Some of the contaminated soil used by Walsh et al. (1995a) was obtained from a coal gasification plant that had maximum PAH concentrations of 3500 mg kg^{-1}. This value is greater then three times the concentration of total PAH found in the PALHI soils. In terms of the number of adducts quantified, after 21 days of exposure, Walsh et al. (1995a) observed 57.7 adducts / 10^9 normal nucleotides, whereas earthworms in the PALHI27 soil had similar average concentration of adducts (64.2 adducts / 10^9 normal nucleotides) after 27 days of exposure. Of interest, the PADCM27 soil had adduct concentrations of approximately double that of the soils used by Walsh et al. (1995a) and the PALHI soils. Both the soil used by Walsh et al. (1995a) and the PALHI soils contained higher PAH concentrations.

Data were analyzed using a general linear model (GLM) procedure in SAS (SAS, 1996) and the means were compared using Duncan's multiple range test. Experiments I and II were analyzed separately and the data from both experiments were also pooled. When pooled, the data were not distributed normally, using a univariate procedure; nor did the data have equal variance, as determined by the Bartlett test. Inferences could not be made on the pooled data, however data for individual experiments were normally distributed and had equal variance, therefore Duncan's test was used to compare mean adduct levels. The Duncan test was chosen because there was one extreme value in experiment one, when included in the data set caused the data to fail the necessary assumptions of the ANOVA. Discarding the value from the data set allowed all ANOVA assumptions to be met. Duncan's test was performed, even with a missing value, by using a harmonic mean for comparing adduct concentrations in experiment I.

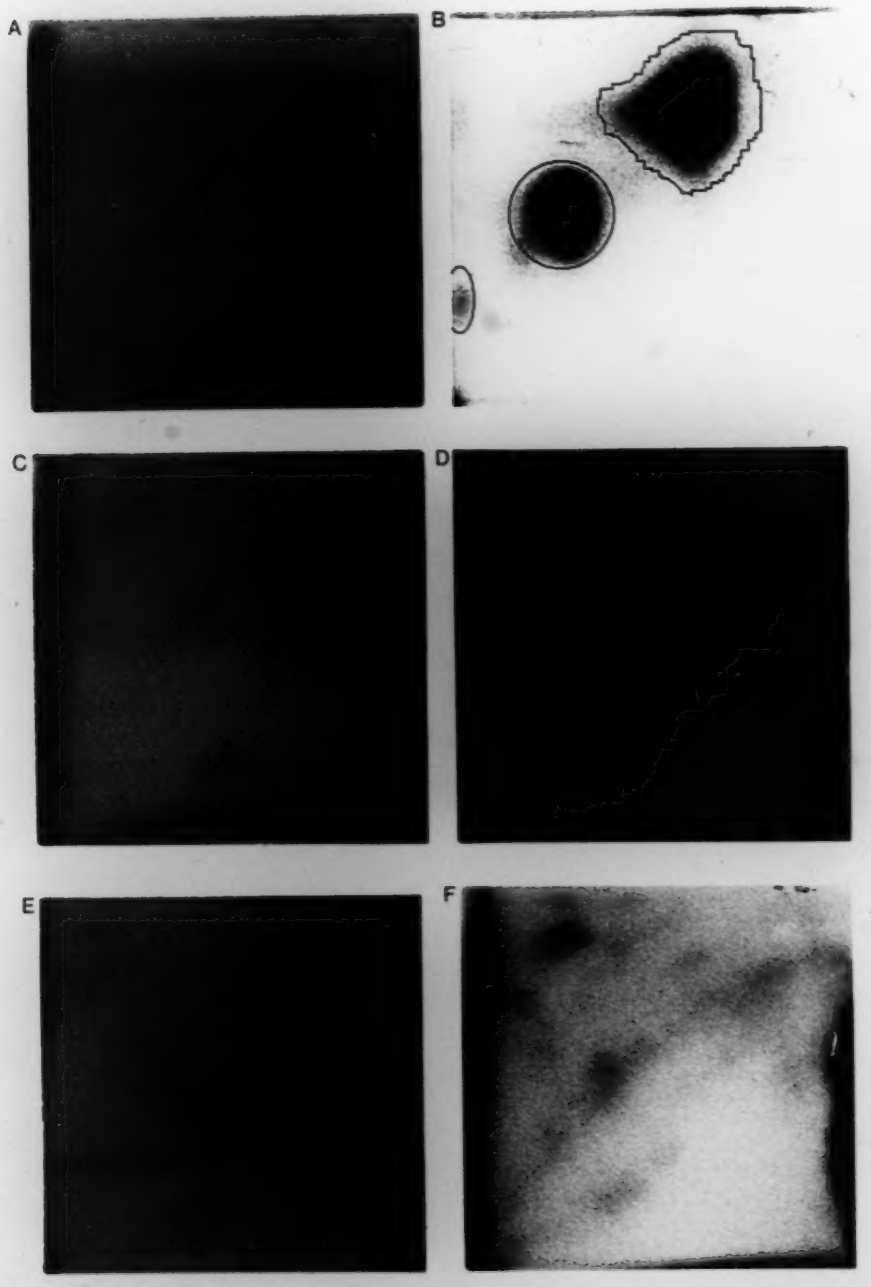


Figure 4.6: Autoradiograms of ^{32}P-postlabeled earthworm DNA samples on PEI cellulose plates. *E. fetida* were exposed to PAH contaminated soils for 27 days, except for the EDM-Bio49 soil, where exposure was 49 days: (A) ATP blank; (B) BaP positive control; (C) BrDCM27; (D) PAHI27; (E) EDMBio14; (F) EDMBio49. All autoradiograms shown were from experiment I, except the ATP blank and BaP control, which were from experiment II.

4.4.2.1 Adduct Experiment I

The soil treatment that resulted in the formation of the highest level of adducts was the PAL soil with only DCM added and exposure time was 27 days (mean = 109.5 ± 28.5) (Figure 4.7). No adducts were detected in the pristine BrDCM soil. Dichloromethane was added to the Bruderheim soil, but there was no creosote contamination present. This suggests that the addition of solvent alone does not contribute to the overall adduct level in *E. fetida*.

The PAL soil with DCM added (exposure = 27 days) had a significantly higher number of adducts ($\alpha = 0.05$) compared to any other soil treatment, except the PAL soil with 1000 mg kg^{-1} of BaP added (exposure = 27 days). The PAL soil with 1000 mg kg^{-1} of BaP (exposure = 27 days) was not statistically different from any treatment. The contaminated soils that had additional BaP added did not cause a statistically significant increase ($\alpha = 0.05$) in the number of adducts formed compared to the pristine control. Even when BaP was added at 10 000 times the Alberta Tier I limit, a statistically significant increase in adduct formation was not seen. In fact the only treatment that had significantly higher numbers of adducts compared to the pristine soil was PAL soil with only DCM added.

In experiment I, increased time of exposure did not produce a significant increase in adduct levels. There were no statistical differences in adduct frequencies in worms exposed to the PAL soil with additional BaP (1000 mg kg^{-1}), whether for 14 or 27 days. Further, adduct frequencies did not significantly increase between worms exposed in the EDM-Bio soil for 14 or 49 days.

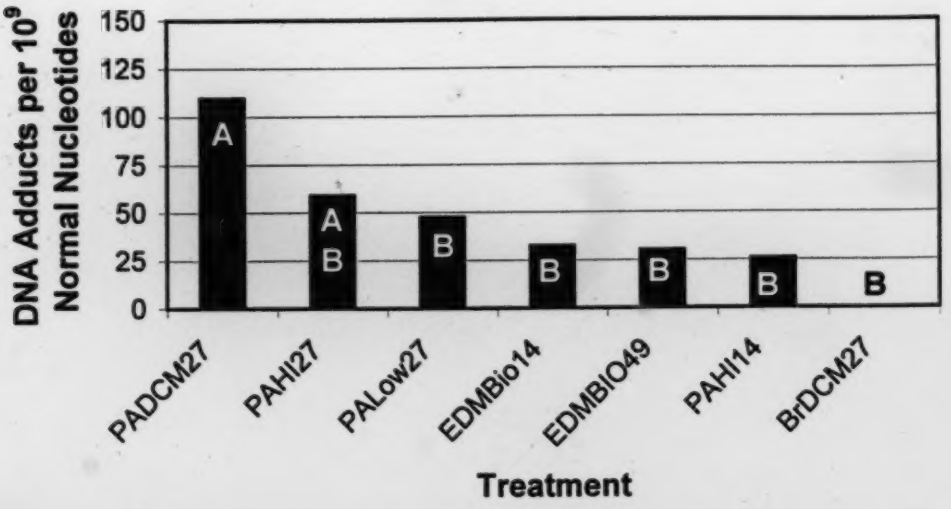


Figure 4.7: Quantification of bulky DNA adducts in *E. fetidia* using a ^{32}P postlabeling assay, experiment I. The values are the mean (n = 3) with one standard deviation. Due to one extreme value, PAHI14 (n = 2). Means with the same letter are not statistically different at the 95% level using the Duncan test.

4.4.2.2 Experiment II

As in experiment I the soil treatment that resulted in the formation of the highest frequency of adducts was the PAL soil with only DCM added and exposure time was 27 days (mean = 84.5 ± 19.0) (Figure 4.8). An average of 7.1 ± 6.2 adducts were detected in the BrDCM27 treatment. Dichloromethane was added to the Bruderheim soil, but there was no creosote contamination present.

The PAL soil with 1000 mg kg^{-1} BaP added (exposure = 27 days) was not statistically different from the PAL soil with only DCM added (exposure = 27 days) treatment, but both treatments were significantly different for all the other treatments. Increased time of exposure in the PAL soil treatments with 1000 mg kg^{-1} BaP added was significant, as there was a statistical difference between the number of adducts measured on day 14 compared to day 27. Walsh et al. (1995a) also showed a time-dependent increase in DNA adduct frequency with *Lumbricus terrestris* exposed to soils contaminated with PAH mixtures. As in experiment I there was no difference between exposure times of 14 and 49 days in the EDM-Bio soil treatments.

A statistical difference in the number of adducts quantified did occur in experiment II between the PAL soil that had 10 mg kg^{-1} of BaP added versus 1000 mg kg^{-1} added. Interestingly, the PAL soil with 1000 mg kg^{-1} BaP added had a higher frequency of adducts compared to the PAL soil with 10 mg kg^{-1} BaP added but no statistical difference was seen with the PAL soil with only DCM added; a soil that did not receive additional BaP.

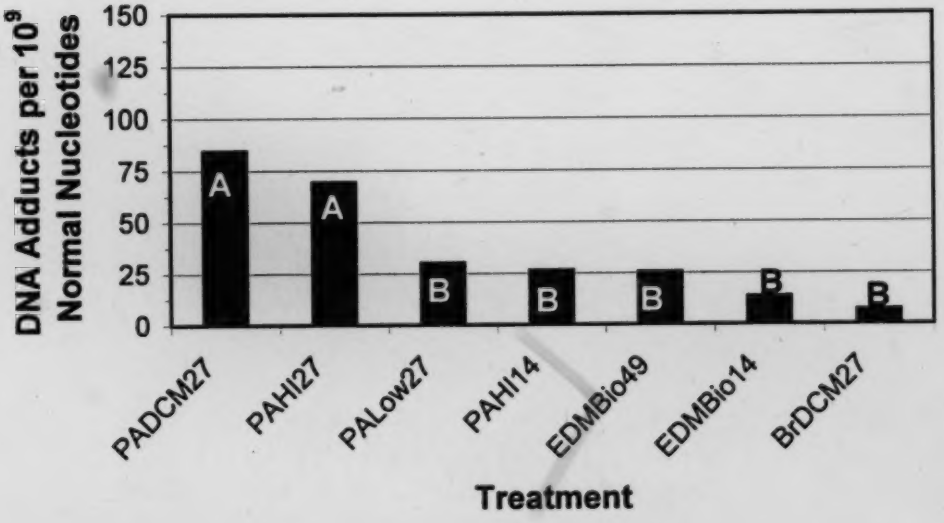


Figure 4.8: Quantification of bulky DNA adducts in E. fetidia using a ^{32}P postlabeling assay, experiment II. The values are the mean (n = 3) with one standard deviation. Treatments with the same letter are not statistically different at the 95% level using the Duncan test.

4.5 Conclusions

Earthworm DNA adduct frequency did not significantly increase when worms were exposed to creosote contaminated soils that had BaP added in a DCM solution, even at 1000 mg kg^{-1}. *Eisenia fetida* exposed to the weathered creosote soil, PADCM, which was treated only with dichloromethane, did have a statistically significant increase in DNA adducts above the level found in the pristine control soil. Potentially, the DCM could have solubilized and redistributed contaminants, increasing contaminant bioavailability. Soils that were biotreated did not have a significantly higher incidence of adducts when compared to the pristine control soil.

Further, increasing time of earthworm exposure to biotreated soils did not result in a significantly higher amount of DNA adducts being formed, even when compared to the pristine soil.

The use of DNA adducts as a measure of subchronic toxicity, did not result in the range of adduct levels that would have been predicted based on chemical data alone. More comprehensive experimentation is required to determine if DNA adducts in earthworms are representative of long term genetic damage. DNA adducts in earthworms might be used as a biomarker of exposure, however, further experimentation would also be required. One area in particular that requires more research is the mode of activation of BaP. Currently, it is unclear how the DNA adducts are formed, whether through conversion by earthworm cytochrome P-450, microbial conversion, or a combination of the former two. Information regarding the activation of BaP would aid in environmental risk management decisions because the actual activation process could then be evaluated and monitored.

4.6 References

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Chapter 5: Synthesis

In combination, chemical and biological testing seem to act synergistically in providing a holistic view of the risk contaminated soils have on *E. fetida*. Implications from the ecotoxicological evaluation of weathered creosote contaminated soils are many fold. Bioassays are useful tools when evaluating environmental contaminant mixtures. Biological assays are able to integrate contaminant / substrate interactions into a measured biological response. The integrated response of the earthworms provided a measure of bioavailability and thus a representation of the risk that creosote contaminated soils pose to the soil biota. Creosote contaminated soils contain a multitude of compounds, and chemical analysis of mixtures can neither predict the toxic interactions between contaminants, metabolites and substrates nor a mixture's ultimate expression as a toxic response. Only bioassays can assess the bioavailability of contaminants, which ultimately relates to the risk of toxicity. Thus biological testing in conjunction with chemical monitoring can aid in the evaluation of risk as well as remediation efficacy. Greater effort is also required for evaluating subchronic toxicity. DNA adduct formation could potentially be used a biomarker of exposure, however comprehensive experiments are required. An ecotoxicological approach, using acute and long term biological testing in conjunction with chemical monitoring, of contaminated sites may result in better risk management decisions, ultimately protecting the environment.

Ecotoxicology investigations should incorporate parameters that are more integrative or informative compared to past practices. The evaluation of a PAH / TEO ratio provides additional information when attempting to evaluate toxicity based on chemical data. The influence of a residual NAPL phase on contaminant availability requires more research effort. Toxicity experiments where BaP is added within an oily carrier could lead to a better understanding of the importance of residual NAPL concentrations on acute and subchronic toxicity. Further, aging of a NAPL, with a known composition, would be helpful in evaluating changes in toxicity

over longer periods of time. Conducting research on the influence of a NAPL on contaminants will ultimately aid in the evaluation and prediction of bioavailability.

Alberta Tier I guidelines for organic contaminants in the soil require reviewing. The EDM-Bio soil was contaminated above Alberta limits for total PAHs and TEO concentrations, however, it was clear that in at least one case, the risk of toxicity to *Eisenia fetida* was low. Lack of observed acute and subchronic toxicity in soils contaminated in excess of the acceptable standards begins to raise questions about the appropriateness of concentration based contaminant limits for all sites. There appears to be situations where the concentration based criteria are overestimating the risk organic contaminants pose to receptors in the environment.

Any evaluation of toxicity testing must also consider the inherent difficulties associated with biological assays. Biological assays often require large investments of time in order to set up and conduct the tests. Earthworm assays are time consuming because each treatment is individually evaluated by hand. Furthermore, standardized earthworms do not exist, making bioassay interpretations more challenging because of inherent genetic variability. Maintaining cultures of earthworms is relatively simple, however, problems can occur because of infestations of mites, fungi, or other worm pathogens. Finally, subchronic bioassay results are difficult to extrapolate to other species. Bioassay results are particularly tempting to apply to humans, however, more research is required to determine whether any bioassay predictions would be valid for humans.

Implications for researchers, regulators, and industry are several fold from the ecotoxicological investigation presented here. Firstly, the PAH / TEO ratio could result in better predictions of potential toxicity and degradability of contaminants. Secondly, ecotoxicological testing could consider the use of water filled pore space (WFP) as a measure of soil moisture. The WFP parameter provides a measure of water content that is not altered due to differences in soil texture or contaminant / water relations. Finally, the use of earthworm-days in both the acute and subchronic toxicity tests was part of a survival based approach. LC_{50} values that are based on survival time endpoints, provide greater insights than time based endpoints. Survival

endpoints are able to integrate concentration data, time, dilutions, and survival. Time based endpoints only include survival data for a specific timeframe.

Soil science has much to offer the discipline of ecotoxicology. Soils are important to consider when evaluating toxicity because the physicochemical soil environment has implications on the biota. Whole soil toxicity testing is therefore required as part of a holistic evaluation of environmental health.

Appendix A: Sample Calculation

A.1 Soil PAH Sample Calculation

X = concentration of an individual PAH, obtained from GC / MSD output (ng μl^{-1})
Y = mass of oven dry soil (g)
DF = Dilution Factor
Clean Solvent refers to the sample extract after passing through a Florisil cartridge
Note: 1 ng μl^{-1} = 1 $\mu g\ ml^{-1}$ and 1 $\mu g\ g^{-1}$ = 1 $mg\ kg^{-1}$

Equation A.1: Conversion formula for GC / MSD output to soil concentration.

$$Soil\ PAH\ Concentration\ (mg\ kg^{-1}) = \frac{50 \cdot X\ (\mu g)\ \cdot DF}{Y\ (g)\ oven\ dry\ soil}$$

$$Soil\ PAH\ Conc = DF \times \frac{X\ \mu g}{(Clean\ Solvent)\ ml} \times \frac{1\ ml\ \ Clean\ Solvent}{Total\ Solvent\ (ml)}$$

$$= \frac{X\ (\mu g) \cdot DF}{Total\ Solvent\ (ml)} \times 50\ ml\ Total\ Solvent$$

$$= 50 \cdot X\ (\mu g) \cdot DF \div Y\ (g\ soil)$$

Table A.1: Sample calculations for replicate one of the EDM-100% soil, Dilution factor = 100. Mass of soil extracted is oven dried weight. PAH values obtained from GC / MSD output.

Compound	PAHs (GC) ng μl^{-1}	Mass of Soil (g)	Soil PAHs (mg kg^{-1})
Naphthalene	0.02	28.25	3.54
Acenaphthalene	0.01	28.25	1.77
Acenapthene	0.46	28.25	81.4
Fluorene	0.56	28.25	99.1
DBT	0.18	28.25	31.9
Phenanthrene	2.16	28.25	382
Anthracene	0.64	28.25	113
Fluoranthrene	1.5	28.25	265
Pyrene	1.2	28.25	212
Benzo(a)anthracene	0.29	28.25	51.3
Chrysene	0.49	28.25	86.7
Benzo(b)fluoranthene	0.15	28.25	26.5
Benzo(k)fluoranthene	0.16	28.25	28.3
Benzo(a)pyrene	0.08	28.25	14.2
Benzo(ghi)perylene	0.02	28.25	3.54
Dibenz(a,h)anthracene	0.01	28.25	1.77
Indeno(1,2,3-cd)pyrene	0.03	28.25	5.31
TOTAL PAHs	7.96	28.25	1407.33
Reported PAH Value			1400

Appendix B: DNA Adduct Quantification

B.1 Materials and Methods

B.1.1 DNA Extractions

Genomic DNA was isolated from *E. fetida* as described by El Adlouni et al. (1995). Evacuated earthworms were washed with tap water, followed by distilled water, then frozen with liquid nitrogen and crushed using a mortar and pestle. Approximately 1 – 2 g of crushed tissue powder was incubated with 10 ml of SET buffer (NaCl 100 mM; EDTA 20 mM, Tris base 50 mM; pH 8.0) and 0.75 ml of SDS (20 %) for 60 min at 65°C. Then 50 µl of proteinase K (Sigma-Aldrich) (10 mg proteinase K in 500 µl of SET buffer) was added to the crushed and incubated tissue reaction mixture, which was further incubated at 37°C for 120 minutes. After the deproteination step, 3.75 ml of potassium acetate (6 M; pH 6) was added to the sample mix and stored at 0°C for 30 minutes to facilitate protein precipitation. Samples were then centrifuged at 10000 g at 4°C for 10 minutes. Sample partitioning to remove unwanted material (mainly proteins and RNA) was then conducted.

An equal volume of buffered phenol (0.1 M Tris-HCl; pH 8.0) was added to the supernatant. The solid material from the centrifuge tube was discarded. The supernatant-phenol solution was gently agitated for 10 min and then centrifuged (10000 g) at 4°C for 15 minutes. The aqueous phase was transferred to a new microcentrifuge tube and the extraction was repeated with different extractants; first with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v:v:v) and then with an equal volume of chloroform:isoamyl alcohol (24:1 v:v). After each extraction, the upper aqueous phase was removed and transferred to a new microcentrifuge tube and the nonaqueous phase and any particulate was discarded. The aqueous phase of each extraction was viscous because of the presence of DNA. Two volumes of ice cold ethanol (95 %) were added to the final aqueous phase of the reaction mixture to precipitate DNA. The mixture was kept at -20°C for 120 minutes. The precipitated DNA was subsequently spooled out of the microcentrifuge tube using the tip of a pipet, and the DNA placed in a new tube. The spooled DNA was

centrifuged (1500 – 2000 g) at 4°C for 15 minutes to form a dry pellet. The dry pellet was then dissolved in 500 µl of SET buffer and the optical density at 260 and 280 nm was determined (Section B.1.2).

Based on the spectrophotometrically determined quantity of DNA, 10 µg of RNase A (Boehringer Manhiem) and 5 units of RNase T1 (previously boiled for 15 minutes) per 150 µg of DNA were incubated at 37°C for 60 minutes, to eliminate RNA. Buffered phenol (equal volume) was added to the sample and then gently agitated for 15 minutes at room temperature. The sample was centrifuged (1500 – 2000 g) at 4°C for 15 minutes. As before, two more extractions were performed, first with an equal volume of phenol:chloroform (1:1 v:v) and then with an equal volume of chloroform . After each extraction the aqueous phase was again transferred to a fresh microcentrifuge tube. Two volumes of cold ethanol (95 %) were added to the final aqueous phase of the extraction mixture. The samples were inverted several times and kept at -20°C for at least 120 minutes. Samples were centrifuged (1500 – 2000 g) at 4°C for 15 minutes. The supernatant was discarded and the remaining pellet was washed three times with ethanol (70 %), aspirating the solution off each time. The pellet was air dried and, then dissolved in SET buffer, at which time the samples were ready to begin the postlabeling procedure (section B.1.3).

B.1.2 DNA Quantification

Quantities and purities of DNA were determined spectophotometrically based on DNA extract values using absorbance data scanning at 220 – 320 nm. First the quantity of DNA was estimated, based on absorbance values at 260 nm; given 1 optical density unit = 50 µg ml^{-1} of double stranded DNA (El Adlouni et al., 1995). The quantity of DNA measured, determined the required quantity of RNase's necessary to fully react with the tissue reaction mixture (Section B.1.1). The purity of DNA was also determined after the DNA extraction using the 260 / 280 nm ratio. A ratio for pure DNA should range between 1.7 to 1.9. Ratios less than 1.7 indicate protein contamination and ratios above 1.9 suggest RNA contamination.

B.1.3 ^{32}P Postlabeling Assay

Reddy and Randerath (1986) developed the general ^{32}P postlabeling method. The method used here is for earthworms and is detailed by Walsh et al. (1995a). The method used here is briefly described, including slight modifications (based on personal communications and recommendations with Dr. D.H. Phillips (UK)). After extraction (Section B.1.1) the DNA samples were aliquoted into usable quantities for the postlabeling procedure (Table B.1), and dried in a speed-vac (SC 100 Savant; Fisher Scientific Co.). Each sample was then reconstituted in 3 µl of digestion buffer (sodium succinate 50 mM, $CaCl_2$ 12.5 mM, pH 6.0). The DNA samples were digested (4 hours) at 37°C in a cocktail of enzymes (micrococcal nuclease 300 U ml^{-1}: 1.5 µl, spleen phosphosdiesterase 2.5 U ml^{-1}: 3.0 µl (Sigma-Aldrich)). After digestion, 0.5 µl was withdrawn for an estimation of total nucleotides in the mix. Nucleotide values were eventually used in the calculation of the number of adducts/10^9 nucleotides. The remainder of the digest (7 µl) underwent a nuclease P1 digestion.

To the digest, 1.5 µl of $ZnCl_2$ (1.3 mM) and 1 µl of nuclease P1 (1 mg/340 µl of 10 mM sodium acetate, pH 5.0 (Sigma-Aldrich)) were added. The samples were incubated at 37°C for 30 min. The reaction was stopped by the addition of 1 µl of Tris base (0.84 M). The samples were then ready to be incubated with carrier free γ-[^{32}P]-ATP (3000 Ci $mmol^{-1}$; NEN Dupont), 100 µCi ATP/sample, and dried in a speed-vac. The dried samples were then incubated at 37°C for 30 min with: 1.76 µl of kinase buffer (170 mM bicine, 85 mM $MgCl_2$, 85 mM dithiothreitol (DTT), 4.3 mM spermidine), 1.34 µl of water, and 1.4 µl of T4 polynucleotide kinase (6 U μl^{-1}) (USB Amersham). All contents were loaded on the thin layer chromatography (TLC) plates.

Resolution of the ^{32}P-labeled adducts was completed using a chromatographic procedure on polyethyleneimine (PEI)-cellulose plates (Polygram CEL 300 PEI plastic backed; Machery-Nagel, Mandel) for the detection of bulky nucleotide adducts. The entire volume, of the labeled sample, was spotted in the middle of a 20 X 10 cm PEI cellulose plate before beginning four dimensional (D) TLC (Figure B.1).

Table B.1: Raw data for DNA quality and quantity, experiment 1.

Earthworm Number	Earthworm Mass (mg)	DNA Obtained (μg)	260 nm / 280nm ratio of DNA	Quantity of DNA used for labeling (μg)
1	250	25.0	1.8	5
2	280	40.0	1.78	5
3	300	42.5	1.74	10
4	280	32.5	1.75	5
5	250	32.5	1.67	5
6	230	32.5	1.7	5
7	270	23.2	1.74	5
8	200	26.8	1.9	5
9	400	15.8	2.0	5
10	400	67.5	1.75	10
11	290	45	1.74	10
12	400	50.0	1.83	10
13	280	35.0	1.75	5
14	200	18.1	1.95	5
15	190	24.7	1.79	5
16	400	57.5	1.79	10
17	300	52.5	1.82	10
18	280	42.5	1.81	5
19	270	10.6	2.0	4.25
20	250	24.2	1.84	4.5
21	250	22.7	1.86	4.5

A 5 X 10 cm wick (Whatman #3 Chr) was clipped on the top of the PEI cellulose plate. After migration of solvent D1 to the wick, the wick was removed and the plate cut in half. The upper portion of the plate was rotated 180° and placed back in the TLC tank to begin the D2 solvent run (5 hr). The plates were rotated 90° for the running of solvent D3 (3.5 hr). Solvent D4 was run in the same direction as D3.

Radioactive adduct spots were identified using autoradiography on the chromatograms. The quantity of adducts were determined using an Instant Imager (Packard Canberra Co.). Knowing the specific activity of the ATP and the decay of ^{32}P, total adduct levels were then calculated by adding the radioactivity of the individual radioactive spots on the chromatograms. The results are expressed as relative adduct levels (adduct/10^9 normal nucleotides) considering 1 μg of DNA is equivalent to 3.03 pmoles of nucleotides (Walsh et al., 1995a).

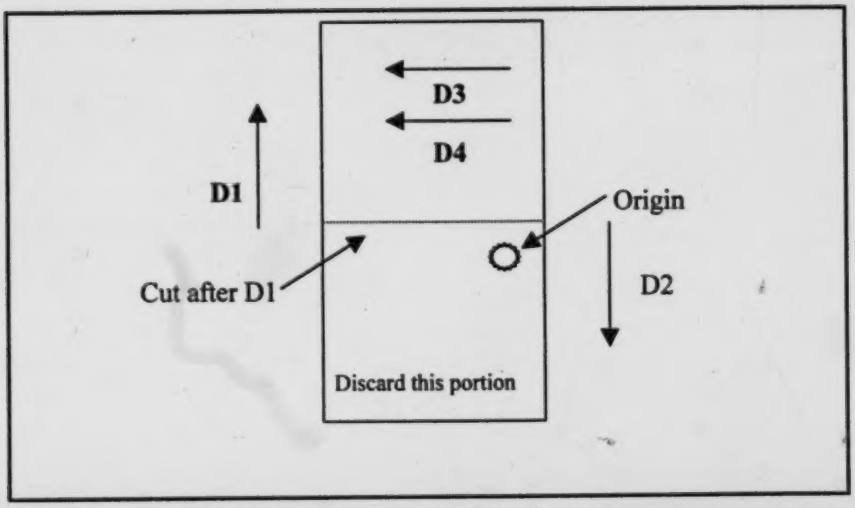

Figure B.1: Representation of the TLC dimensions on PEI cellulose plates after spotting the entire sample volume of at the origin. Solvent mixtures were: D1: 1 M sodium phosphate buffer, pH 6.0; D2: 8.5 M urea, 8 M lithium formate, pH 3.5; D3: 2.0 M lithium chloride, 1.0 M Tris, 8.5 M urea, pH 8.0; D4: 1.7 M sodium phosphate buffer, pH 6.0, adapted from Walsh et al. (1995b).

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